Understanding the chicken intestinal microbiome: towards a rational approach to feed-based interventions

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Abstract

The intestinal microbiome has been identified as a key component in animal health. This has led to the development of direct-fed microbials which aim to optimise the composition of the intestinal microbiome in order to improve production or disease resistance. In the case of broiler chickens reared for meat, direct-fed microbials could replace antibiotics which have been a mainstay of growth promotion and disease prevention in the industry. There is limited knowledge of normal intestinal microbiome development in broilers regarding common taxa and key markers of microbiome maturation. Such information could be used to identify biomarkers of development, better define success of probiotic interventions, optimise timing of interventions or identify probiotic candidates. The first aim of this thesis was to re-examine microbial colonisation and succession of the ileal and caecal microbiome over a typical broiler lifespan using modern next generation sequencing techniques. The microbiome at hatch was composed of environmental taxa such as Enterobacteriaceae. The first commensals to colonise the caecum were Lachnospiraceae and Bifidobacteriaceae followed by Bacteroidaceae and Ruminococcaceae. A mature microbiome formed between 14 and 21 days post hatch (d.p.h). In the ileum, Enterococcaceae were a major constituent of the microbiome but were replaced by Lactobacillaceae from 14 d.p.h. The abundance of Candidatus *Arthromitus*, an immunogenic segemented filamentous bacteria, was highest between 7 and 21 d.p.h. Other taxa such as *Turicibacter* and *Romboutsia* were present from 14 d.p.h. These developmental stages were used to assess the success of two experiments which observed the impact of interventions on intestinal microbiome development and composition. The first intervention was a topical application of diluted caecal contents to eggs in an attempt to transplant a normal microbiome to chicks. This resulted in accelerated intestinal microbiome development as evidenced by increased and earlier colonisation of Lachnospiraceae and Ruminococcaceae in the caecum and Candidatus *Arthromitus* in the ileum. However, the treatment failed to transplant some key taxa such as Bacteroidaceae and Lactobacillaceae. The second intervention consisted of a multistrain *Bacillus amyloliquefaciens* probiotic included in the feed. No significant differences in microbiome composition were found between treated and control chicks. Overall, these results provide a framework for assessing the success of interventions on the microbiome. Microbiome transplants which expose chicks to a normal microbiome at hatch are likely to be more successful for altering microbiome colonisation and composition than direct-fed microbials.

Publications

Publications included in this thesis

Chapter 2

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Abbreviations

ASV	Amplicon sequence variant
BA	*Bacillus amyloliquefaciens*
BHI	Brain heart infusion
b.p	Base pairs
BSH	Bile salt hydrolases
CCEP	Cultured competitive exclusion product
CD	Cluster of differentiation
CE	Competitive exclusion
CEC	Competitive exclusion culture
CFU	Colony forming units
CT	Caecal tonsil
Ct	Cycle theshold
CR	Conventionally reared
ddNTP	Dideoxynucleotide triphosphates
DGGE	Denaturing gradient gel electrophoresis
d.i	Days of incubation
dNTP	Deoxynucleotide triphosphates
DNA	Deoxyribonucleic acid
d.p.h	Days post-hatch
F/B ratio	Firmicutes:Bacteroidetes ratio
FCR	Feed conversion ratio
FDR	False discovery rate
FISH	Fluorescence *in situ* hybridisation
FPD	Faith's Phylogenetic Diversity
FTU	Phytase units
GALT	Gastrointestinal-associated lymphoid tissue
GC	Germinal centre
GF	Germ-free
H&E	Haematoxylin and eosin
IEL	Intraepithelial lymphocytes
IL	Interleukin
NDA	Not differentially abundant
NGS	Next generation sequencing

NK cells	Natural killer cells
OCT compound	Optimal cutting temperature compound
OTU	Operational taxonomic unit
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PMA	Propidium monoazide
PP	Peyer's patches
QIIME2	Quantitative Insights In Microbial Ecology version 2
qPCR	Quantitative PCR
RBB	Repeated bead-beating
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SCFA	Short chain fatty acid
SD	Shannon diversity
SFB	Segmented filamentous bacteria
Std Dev	Standard deviation
TCR	T-cell receptor
TGGE	Temperature gradient gel electrophoresis
T-RFLP	Terminal restriction fragment length polymorphism
tRNA	Transfer ribonucleic acid
TSS	Total suspended solids
UniFrac	Unique Fraction
ZSBC	Zymobiomics standard bacterial community

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Chapter 1

Literature Review

1.1 Introduction

1.1.1 The poultry industry

Between 1961 and 2001, global average annual meat consumption per capita nearly doubled from 23.1kg to 42.20kg (Sans and Combris, 2015). Much of this increase has been provided by a growing poultry industry which has intensified and industrialised production to meet demand whilst reducing prices for consumers in both developed and developing countries. The poultry industry is broadly divided into sectors for meat and egg production. Each sector uses a specific type of hybrid chicken breed termed broilers, for meat production, and layers, for egg production. The broiler industry produces far more birds, with 21 million broiler chicks placed by UK hatcheries per week in 2018 compared to 800,000 layer chicks (Department for Environment, Food Rural Affairs, 2019). The industrialisation of poultry production has led to separation of each stage at independent sites. Breeder flocks, constituting the genetic elite of the national chicken population, are kept exclusively to provide fertile eggs. Eggs are transported to hatcheries, which may be on the same site, where they are incubated in batches until hatch. After hatch, chicks are sold as 'day-old chicks', which may be up to 72 hours old, to finishers where they remain until slaughter at around 42 days old. Much of the poultry industry is vertically integrated, with the same company owning breeder flocks, hatcheries, finishers, feed mills and processing plants. This vertical integration has lead to an efficient industry that produces approximately 82 million broilers a month for slaughter (Department for Environment, Food Rural Affairs, 2019).

However, industrialisation has brought detrimental effects on the health and welfare of

poultry. Increased stocking densities lead to increased mortality, behavioural disturbances and disease (Hall, 2001). Such conditions inducing physical and psychological stresses can increase the susceptibility to infectious disease (Humphrey, 2006). As a result, poultry flocks are exquisitely sensitive to the presence of enteric pathogens. While many can be controlled using vaccination and biosecurity, pathogens such as *Campylobacter* remain rife within the UK chicken population (Veterinary Record, 2015). In a bid to control enteric pathogens and their negative effects on production, prophylactic use of antibiotics in the poultry industry was widely adopted and used as a growth promoter (Dibner and Richards, 2005). However, the indiscriminate use of antibiotics has led to a rise in antimicrobial resistance with implications for public health. In order to combat this threat to public health, the European Union enacted a ban on the use of antibiotic growth promoters in 2006 (Castanon, 2007). As a result, this crutch of the poultry industry must be replaced with alternatives such as probiotics that maintain production by promoting gut health.

1.1.2 Probiotics and the microbiome

Probiotics have been widely used in the poultry industry as feed additives supported by evidence showing that their use can improve growth performance and reduce the impact and incidence of gastrointestinal disease (Kabir, 2009; Nava *et al.*, 2005). However, the rationale for use of probiotics must take into account the wider microbiological picture. Any probiotic included in feed must coexist and compete with bacteria and other microbes that are already inhabiting the gastrointestinal tract, collectively termed the microbiome. Understanding the chicken intestinal microbiome is an essential step in developing an effective approach to probiotic use. An understanding of the normal succession and development of the chicken intestinal microbiome will allow for identification of significant milestones in development which can be used as parameters for good intestinal health. Additionally, the use of 16S ribosomal RNA (rRNA) gene sequencing can be used to identify suitable taxa for a new generation of probiotics. Current probiotics are limited to a few genera of easily cultured bacteria such as *Lactobacillus* and *Bacillus* but the development of new culture techniques may allow other and more diverse taxa to be brought into probiotic production.

The first use of the word microbiome has been attributed to the microbiologist Joshua Lederberg who used it to describe

> "...the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space..." (Lederberg and McCray, 2001).

Since then, 'microbiome' has been used as an all-encompassing term for the fields of microbial proteomics and metagenomics, as well as the phylogenetic and phenotypic classification of microbial communities. Microbiome has come to be synonymous with the terms microbiota, microflora and microbial community which were used to describe earlier attempts to elucidate the composition of a variety of microbiomes.

Culture used to be the principal method of studying microbial communities. However, the fastidious nature of some bacteria, amongst other difficulties with culture, limited microbiologists' understanding of the interactions between hosts and their microbial communities. Molecular techniques such as early sequencing technology and fluorescent *in situ* hybridisation (FISH) increased the understanding of microbiome structure and diversity but were limited by low throughput and current knowledge of DNA sequences respectively. However, as DNA sequencing became cheaper, faster and more efficient, the scope and detail of microbial community analysis increased, providing a wealth of information about the ecology of microorganisms in different environments.

All methods used for studying microbiomes depend on three central concepts:

1. Advances in DNA sequencing, initially Sanger sequencing followed by 454 pyrosequencing and Illumina sequencing.

2. The adoption of 16S rRNA genes as the standard marker for the phylogenetic classification and identification of bacteria.

3. The polymerase chain reaction (PCR) which allows for the amplification and subsequent sequencing of 16S rRNA genes from environmental samples.

1.1.3 DNA sequencing

Following Watson and Crick's paper on the molecule's three-dimensional structure, early attempts to sequence DNA were aimed at naturally abundant RNAs such as bacterial rRNA or transfer RNA (tRNA) (Heather and Chain, 2016). The first whole nucleic acid sequence was of alanine tRNA from *Saccharomyces cerevisiae* and was published in 1965 by Holley *et al.* who analysed the nucleotide composition of RNA fragments degraded by treatment with selective ribonucleases. For another 12 years, various sequencing techniques were tried and altered until Frederick Sanger provided the next breakthrough in 1977. Sanger sequencing, also known as Sanger's chain-termination or dideoxy technique, used dideoxynucleotide triphosphates (ddNTPs) which lack a 3' hydroxyl group and consequently cannot bind to a 5' phosphate of the subsequent deoxynucleotide triphosphate (dNTP). By including a small amount of radiolabelled ddNTPs in a standard DNA extension reaction, a mixture of DNA fragments of varying lengths is produced as DNA chains ending with a ddNTP will not be further extended. Each template DNA sequence requires four reactions to be run, one with each of the ddNTPs (ddATP, ddTTP, ddGTP and ddCTP). The products of these four reactions are loaded into separate lanes of a polyacrylamide gel, separated by electrophoresis and imaged using autoradiography. The autoradiographs can be read to decipher the nucleotide sequence of the original DNA template. Further refinements such as using fluorescent labels instead of radiolabels and the development of capillary electrophoresis allowed for the automation and miniaturisation of sequencing leading to reduced costs and increased throughput. Second generation sequencing techniques, like pyrosequencing, began development in 1988 but wouldn't become commercially available until 2005 (Heather and Chain, 2016).

The development of sequencing released DNA's potential as a historic record of genetic relations between organisms and opened the field of molecular evolution. Biologists could begin to classify relationships based on genetic markers rather than morphological, physiological or biochemical characteristics. The first step would be to find a genetic sequence which was present in all organisms and acted as a reliable molecular clock over a time span

of hundreds of millions of years. The results would overturn the widely-accepted division of life into prokaryota and eukaryota as well as other divisions within the bacterial world.

1.1.4 16S rRNA genes

The idea of a molecular clock was first proposed by Emile Zuckerkandl and Linus Pauling in relation to the structure of haemoglobin. They proposed that differences in the protein sequence (and thus the DNA sequence) could be used to estimate the time since evolutionary divergence of different species (Zuckerkandl and Pauling, 1962). This idea was extended to other proteins and appeared, as a proof of concept, to support the phylogeny already established by morphology. However, the accepted classification of bacteria didn't hold up when examined from the point of view of molecular evolution. In 1977, Woese and Fox published a paper suggesting that, based on their observations of the structure of rRNA, the division of life into nucleated and non-nucleated cells was inherently superficial. They proposed that life was grouped into three 'urkingdoms' (now called domains): Eubacteria, made up of typical bacteria; Archaebacteria, the methanogenic bacteria and Urkaryotes, a hypothetical group of organisms representing the ancestors of eukaryotes. They argued that the sequences of ribosomes from Archaebacteria were as distinct from Eubacteria as Eubacteria were from Urkaryotes and so were deserving of their own urkingdom (Woese and Fox, 1977). Although the idea was not widely accepted initially, Woese continued to work using rRNA as a phylogenetic marker eventually publishing "Bacterial Evolution" in 1987 in which he described the ideal characteristics of a molecular clock and how rRNA genes (also referred to as rDNA) fit these characteristics. According to Woese a molecular clock must:

1. Exhibit clock-like behaviour – in the molecular sense this refers to a genetic sequence which accumulates base sequence changes at a constant rate. Most importantly these changes must not be selected or else they will accumulate and obscure random changes.

2. Have adequate temporal range – change slowly enough to span the full evolutionary

spectrum being measured.

3. Have more than one domain – Woese defines domains as regions which are evolutionary independent of each other. Non-random change in one domain will not affect others. If one domain is affected by evolutionary changes other domains can be analysed instead. Another consideration is the nucleotide length of the gene. A very long gene will yield good statistical data but, at the time, would take much longer to sequence than a shorter gene (Woese, 1987).

Olsen *et al.* (1986), also working on rRNAs, identified six features of the molecules that make them suitable for use as molecular clocks:

1. Since rRNAs are essential for protein synthesis they are present in all organisms.
2. The structure of rRNAs is highly conserved and they are therefore easily identified.
3. The nucleotide sequence is formed of hypervariable regions flanked by conserved regions. These conserved regions allow for the alignment of variable regions and ensure that homologous nucleotides are being compared between species. They also provide convenient sites for primer attachment.
4. Each cell contains many rRNA molecules so they are easy to recover.
5. Sequences are long enough to allow statistically significant comparisons.
6. rRNA genes are not transferred laterally between bacteria so they represent true evolutionary relationships between organisms.

Woese also noted that different positions on rRNA genes change at different rates, allowing a full range of the evolutionary spectrum to be observed using one molecule (Woese, 1987).

There are three rRNA genes common to all organisms: 5S, 16S, and 23S. Initially, 5S was used because its short length made it easier to sequence although consequently it was less useful statistically. As sequencing technology advanced to allow longer reads, partial

16S sequences were used. Currently, the 16S rRNA gene is the standard phylogenetic marker used to identify and classify organisms in studies of microbial ecology and diversity. The use of the 16S rRNA gene allowed for the phylogenetic classification of culturable bacteria which could yield sufficient DNA for sequencing. However, DNA recovered from environmental samples was often not present in large enough quantities to be sequenced. What microbial ecologists needed was a method for artificially replicating DNA in the laboratory. This finally became available in 1983 with the development of PCR.

1.1.5 The polymerase chain reaction

Before 1983, some methods of synthesising small amounts of single-stranded DNA using DNA polymerase were available in laboratories. Kary Mullis further developed these techniques by adding two oligonucleotide primers which were complementary to opposite ends of the sense and antisense strands of a DNA template so allowing for targeted and repetitive synthesis of a specific section of DNA. Further modifications to the original procedure have converted PCR into an essential tool in a wide variety of fields including microbial ecology (Bartlett and Stirling, 2003). Before the advent of PCR, DNA extraction from environmental samples could not yield sufficient 16S rRNA genes for a population analysis. Studies relied on the ability to culture bacteria and then use 16S rRNA genes from each culture to identify the bacteria. PCR offered a way, once the correct primers had been identified, to amplify the 16S rRNA genes of all bacterial species in a sample to a level where they could be sequenced. Although PCR is not a perfect method, its ability to amplify DNA signatures from uncultured organisms has added a whole new dimension to the analysis of microbial communities.

Before reviewing previous work on the composition of the chicken intestinal microbiome, it is worth examining techniques for the extraction and amplification of DNA from samples as well as sequencing and analysis of 16S rRNA genes. This will allow us to scrutinise the methods and materials used in previous studies and analyse the quality of their results. Since microbial ecology is a relatively new field using new technology, techniques developed in the past have been subsequently revealed to confer a bias to certain bacterial taxa.

1.2 Obtaining Sequences From Microbial Communities

A well-planned and justified methodology and analytical pipeline are essential for obtaining good quality results from 16S rRNA gene amplicon sequencing studies. This section aims to review the literature regarding sample collection, storage, processing and analysis to inform the methodology of the subsequent studies. A summary of a typical 16S rRNA gene amplicon study including steps from sample collection to data analysis is provided in Figure 1.1.

1.2.1 Sampling the community

The way in which DNA from a sample is handled, extracted and amplified can introduce biases into the analysis of a microbial community. The techniques used will differ depending on the environmental matrix being sampled. The following review will focus on the sampling of intestinal contents or faeces.

Sample collection

The most important factor to consider while collecting samples is contamination. Possible sources of contamination include the environment, the researcher and any equipment used. Each source of contamination must be identified and minimised before sampling. The majority of studies do not give many details describing how the gastrointestinal tract was excised and treated before DNA extraction. Some groups have excised the gastrointestinal tract and transferred them to individual sterile bags to be kept on ice until contents can be sampled in a clean environment (Amit-Romach *et al.*, 2004; Ballou *et al.*, 2016; Park *et al.*, 2016; Zhu *et al.*, 2002). The opening of the coelom need not be done using strict aseptic protocol as the gut will act as a physical barrier to contamination. However, it is worth maintaining clean conditions and avoiding unnecessary contact between the gastrointestinal tract and contaminating surfaces before individual packaging. To avoid spillage of gut contents during transport, the excised ends of the gastrointestinal tract should be clamped with forceps (Zhu *et al.*, 2002). Content sampling should be conducted as soon as possible

after excision of the gastrointestinal tract. This should take place in an area which can be treated with UV light to destroy contaminating DNA between samples. Equipment such as scalpels and forceps should be cleaned and soaked in 30% bleach for 30 minutes before autoclaving to ensure that DNA has been removed (Pedroso *et al.*, 2016).

During the sampling of gut content, general aseptic protocols should be followed. These may include measures such as:

- The use of personal protective equipment to minimise contamination from skin, clothing and hair.
- The use of 70% ethanol to clean work benches and the surface of the gut before sampling.
- Working within a fume hood which can be treated with UV light between samples.

Non-viable cell exclusion

Since DNA can persist within a cell after death and be amplified by PCR (Josephson *et al.*, 1993) it is important to differentiate viable from non-viable cells during microbial community analysis. This is especially important when studying the effect of probiotics supplemented in feed since any fed bacteria will contribute to DNA in a sample but may no longer be viable. Failure to exclude non-viable cells can skew results (Rogers *et al.*, 2013). One option is to analyse RNA instead of DNA since only metabolically active cells will produce RNA. However, difficulties with RNA extraction, molecule instability and physiological variations limit its use (Desneux *et al.*, 2015). Other methods include flow cytometry and analysis of substrate responsive and non-responsive cells, yet each of these techniques also introduces its own biases and limitations (Nocker *et al.*, 2007).

Pre-treatment of samples with propidium monoazide (PMA) has been suggested as a possible method for excluding non-viable cells from PCR amplification. PMA is a membrane impermeant dye which only penetrates cells whose membranes have been compromised. When exposed to light, the azide group covalently binds to DNA and will block amplification during PCR (Nocker *et al.*, 2007). The technique has been used to successfully differentiate

live from dead bacteria in environmental matrices such as water, food and sputum (Erkus *et al.*, 2016; Nocker *et al.*, 2007; Rogers *et al.*, 2013). Faeces and intestinal contents pose a challenge compared to these matrices because turbidity of intestinal content might block photoactivation of PMA and organic matter may affect the efficiency of PMA penetration of non-viable cells (Desneux *et al.*, 2015).

Optimisation of PMA pre-treatment is essential when working with difficult substrates. Several studies have focused on PMA pre-treatment of biosolids, a term used to describe sewage which has been treated and discharged from a treatment plant (van Frankenhuyzen *et al.*, 2011). Optimisation should focus on three factors: PMA concentration, photoactivation time and amount of total suspended solids (TSS) (Bae and Wuertz, 2009; Desneux *et al.*, 2015). Each of these three factors will introduce its own bias when altered too much, for example, increased PMA concentrations will encourage penetration of living as well as dead bacteria, increased light exposure will promote non-selective PMA binding and excessive sample dilution can lead to inadequate DNA yields (Bae and Wuertz, 2009; van Frankenhuyzen *et al.*, 2011). If the TSS is less than or equal to 2000mg/l, a PMA efficiency of 99% can be achieved in biosolids using a PMA concentration of 100mM and 5 minutes of light exposure (Taskin *et al.*, 2011). Similarly, 100mM of PMA and 10 minutes of light exposure was shown to be effective in biosolid samples with a TSS of up to 1000mg/l (Bae and Wuertz, 2009).

Another possible complication of using PMA pre-treatment in samples destined for 16S rRNA gene sequencing is suppression of amplication of short DNA targets. Luo *et al.* (2010) targeted the V3 region of the 16S rRNA gene which is roughly 190 base pairs (b.p) long. They found that PMA pre-treatment was not sufficient to exclude DNA from non-viable cells. This was overcome by using a two-step PCR in which the entire 16S rRNA gene is amplified followed by a second PCR to target the V3 region . Further data relating to the effectiveness of PMA pre-treatment when amplifying longer variable regions is not available.

Sample storage

If DNA extraction can't be carried out on fresh samples then samples will have to be stored. The recommended method is freezing at -80°C in ambient atmosphere (Thomas *et al.*, 2015). Samples snap frozen in liquid nitrogen and those frozen at -80°C were similar in the taxonomy and relative abundance of bacteria detected when compared with fresh samples although there was an increased relative abundance of Firmicutes and decreased relative abundance of Bacteroidetes in snap frozen and -80°C frozen samples (Fouhy *et al.*, 2015). Improved DNA extraction from Gram-positive bacteria such as Firmicutes due to cell wall damage caused by freezing could explain this shift in Firmicutes:Bacteroidetes (F/B) ratio in frozen samples (Bahl *et al.*, 2012). However, a change in the F/B ratio is not always observed in studies examining different methods of sample storage (Lauber *et al.*, 2010).

1.2.2 DNA extraction

In terms of microbial ecology, the aim of DNA extraction is to produce a representative sample of all the microbes within a sample. No single method is perfect and while adding further steps of purification may yield a more suitable product for PCR, DNA will be lost with each subsequent step.

The first step of DNA extraction is the release of DNA from bacterial cells by lysis. The method of lysis can introduce severe bias as a cell's susceptibility to rupture depends on the structure of its outer membranes and cell wall (Stackebrandt *et al.*, 1999). Gentle lysis methods introduce a bias against Gram-positive bacteria due to their sturdier cell walls. Equally, vigorous lysis may damage DNA from fragile bacteria. This sheared DNA is more likely to produce PCR artefacts such as chimeras and heteroduplexes (Röling and Head, 2004; Stackebrandt *et al.*, 1999). Lysis techniques are based on either mechanical, chemical or enzymatic degradation of bacterial cells. The success of chemical and enzymatic methods varies greatly depending on the environmental matrix which may not have an optimal pH or ionic conditions (Milling *et al.*, 2004). The most commonly used mechanical technique is bead-beating in which the sample is vigorously shaken in the presence of 0.1mm to 0.5mm

diameter beads. As mentioned previously, this mechanical disruption is not specific to bacterial cell walls and membranes leading to DNA shearing. The amount of DNA shearing is dependent on a variety of factors such as the time and intensity of beating; the size of the beads and the bead to suspension ratio. A repeated bead-beating (RBB) protocol has been developed which can help limit DNA shearing. A first round of bead-beating is conducted to lyse Gram-negative bacteria. The lysate is removed before a second round of beat-beating is conducted to lyse Gram-positive bacteria and Archaea. This minimises DNA damage by limiting exposure of DNA from fragile bacteria to unnecessary beating (Yu and Morrison, 2004). When compared to other methods, RBB was found to have a superior DNA extraction efficiency and provide the best phylogenetic diversity, especially with respect to Archaea and Gram-positive bacteria (Claassen *et al.*, 2013; Salonen *et al.*, 2010).

After cell lysis the DNA must be extracted from the mixture of cell debris and environmental matrix. A wide variety of commercial kits are available and each can introduce its own bias in terms of phylogenetic diversity and quality of DNA extracted (Claassen *et al.*, 2013; Mirsepasi *et al.*, 2014). This makes the comparison of studies examining the chicken gastrointestinal microbiome difficult as many will have used different DNA extraction techniques. Alternatively, if a commercial kit is not available, Apajalahti *et al.* (1998) described a method for lysis and extraction of DNA from ileal and caecal samples with reported cell lysis rates of >95% and >99% respectively.

The final step of DNA extraction is purification which aims to remove PCR-inhibiting substances such as Dnases, polysaccharides and proteases which can interfere with the amplification of DNA (Wilson, 1997). PCR inhibitors are present in chicken faecal and caecal samples with a higher level detected in caecal samples (Rudi *et al.*, 2004). Commercially available DNA extraction kits contain steps which will help remove PCR inhibitors from samples however there are additional measures which can be taken. The addition of non-acetylated bovine serum albumin to faecal and caecal samples has been shown to partially overcome this inhibition, while polyethylene glycol also facilitates PCR of faecal, but not caecal, samples (Rudi *et al.*, 2004).

Once DNA extraction is complete, the quality of extracted DNA can be estimated using agarose gel electrophoresis and the quantity using a spectrophotometer such as NanoDrop (Gillings, 2014; Schokker *et al.*, 2015; Stanley *et al.*, 2013*b*).

1.2.3 PCR amplification of 16S rRNA genes

In any PCR, the reaction vessel must contain the following ingredients:

- A thermostable DNA polymerase.
- A forward and reverse oligonucleotide primer pair.
- Free dNTPs.
- Mg^{2+} ions.
- Template DNA (from the extracted DNA) (Röling and Head, 2004).

The reaction vessel completes timed cycles of different temperatures at which different stages of DNA amplification take place. Each cycle has 3 stages:

1. Denaturing of double-stranded DNA to form single-strands at 94 – 96°C.
2. Annealing of primers (forward to the sense strand, reverse to the antisense strand) at a variable temperature.
3. Extension of the DNA from the primers by DNA polymerase at 68 – 78°C (Röling and Head, 2004).

The oligonucleotide primers are the most controllable variable of any PCR and can be a source of bias. Even if Firmicutes are the most abundant phylum in the environmental sample they will not be found on subsequent analysis if the primers don't successfully bind to Firmicutes DNA. As such, primer design is a major component of any microbial community study.

Primer design

As mentioned before, the 16S rRNA gene is present in all organisms from the three domains: Archaea, Bacteria and Eukaryota. The nucleotide sequence can be divided into 9 hypervariable regions (termed V1 – V9) flanked by conserved regions (Chakravorty *et al.*, 2007). Primers that target the 16S rRNA gene are designed to bind to the conserved regions allowing for replication of the hypervariable regions which can then be analysed depending on the goals of the study. A wide variety of primers can be chosen depending on whether researchers wish to amplify the entire gene or just specific hypervariable regions.

There is no established nomenclature for primers, but two systems are prevalent providing a short or a long name. The long name uses the system proposed by Alm *et al.* (1996) in which each primer is named via seven key features separated by hyphens, e.g. S-D-Bact-0008-a-S-20. The short name consists of a number corresponding to the first base of the target sequence on the 16S rRNA gene, followed by either F (for forward) or R (for reverse). For example, the universal primers that amplify the entire 16S rRNA gene are 8F and 1492R (Lu *et al.*, 2003; Gong *et al.*, 2007). Short names may be followed by a -GC, which shows the presence of a GC-clamp used for denaturing and temperature gradient gel electrophoresis (DGGE and TGGE). The numbering system in both methods corresponds to the base from the 16S rRNA gene of *Escherichia coli* unless otherwise specified (Alm *et al.*, 1996; Brosius *et al.*, 1978). The hypervariable regions have been identified as spanning the following bases: 69 – 99, 137 – 242, 433 – 497, 576 – 682, 822 – 879, 986 – 1043, 1117 – 1173, 1243 – 1294 and 1435 – 1465 for V1 to V9 respectively. This can be used to identify the hypervariable region which primers target for example 986F and 1401R will amplify the V6, V7 and V8 regions (Chakravorty *et al.*, 2007).

The best primers to use will be defined by the aims of the experiment and methods which will be used. Typically, techniques for fingerprinting microbial communities like DGGE and terminal restriction fragment length polymorphism (T-RFLP) use DNA fragments of no more than 500 b.p (Schmalenberger *et al.*, 2001). Modern sequencing techniques are also limited by read length with higher throughput techniques often producing shorter read

lengths. 454 pyrosequencing produces read lengths of up to 700 b.p while Illumina platforms only provide reads of approximately 300 b.p. Since the 16S rRNA gene is approximately 1500 b.p long, we must select which variable region to use in our analysis. Not all variable regions are of equal merit for taxonomic classification of bacteria. For example, it has been reported that V2 was unable to distinguish between common staphylococcal and streptococcal pathogens but provided the best region when analysing differences between mycobacterial species (Chakravorty *et al.*, 2007). The same study concluded that regions V2 and V6 have the maximum nucleotide heterogeneity and therefore are the best regions for discriminating between a selection of 110 bacterial species (Chakravorty *et al.*, 2007).

When designing primers there are two viable options. It is possible to review the literature and reuse primers which have been previously validated. However, it's important to consider whether these primers are still valid as new additions to 16S rRNA databases may reveal that previous primers are not as specific as once thought. Alternatively, software such as ARD or PRIMROSE can be used to design primers according to sequences for bacteria which appear in online databases of 16S rRNA gene sequences (Röling and Head, 2004).

PCR artefacts

The amplification of DNA is a laboratory imitation of DNA replication and so is subject to the same errors. Since DNA polymerase is not 100% accurate, point mutations and deletions can occur which will alter the replicated sequence from its original template. This error will then be amplified and may introduce a false element of diversity into the analysis. The observed error rate for *Taq* DNA polymerase during PCR depends on the reaction conditions, and varies between one error per 10^4 and 10^5 b.p (Hestand *et al.*, 2016). The error rate also differs between DNA polymerases, for example using *Pfu* instead of *Taq* DNA polymerase leads to a 10-fold improvement in the error rate (Hestand *et al.*, 2016; McInerney *et al.*, 2014). Reading errors will also increase with the number of PCR cycles so cycle number must be kept to a minimum.

The formation of heteroduplexes during PCR also presents a problem. Heteroduplexes

are double-stranded DNA molecules formed of single strands from different sources. As PCR progresses, the primer:template ratio decreases and can reach a point where primer annealing is no longer favoured. This leads to hybridisation of heterologous template DNA and the formation of heteroduplexes. Heteroduplexes can increase the number of bands if the sample is analysed using DGGE or TGGE and introduce biases during the construction of clone libraries (Thompson *et al.*, 2002). Various methods for reducing heteroduplex formation have been proposed. These include the addition of more *Taq* polymerase after the 27th cycle, limiting the cycle number (Michu *et al.*, 2010) and 10-fold dilution of the PCR product followed by three cycles of re-amplification (Thompson *et al.*, 2002).

Chimeras are more troublesome artefacts. They occur when an incompletely synthesised 16S rRNA gene fragment from the extension phase binds to a homologous fragment during the annealing phase to form a heteroduplex. The incomplete fragment then acts as a primer for extension, creating a chimera of two 16S rRNA genes from different species. This is then amplified and can be easily confused as originating from a new, but unfortunately non-existent, bacterial species. Amplification of 16S rRNA genes is prone to the formation of chimeras because of the conserved regions of the gene. PCR amplification of 16S rRNA genes has produced between 5.4 and 8.6% chimeras although reported chimera rates can be as high as 45% (Haas *et al.*, 2011; Wintzingerode *et al.*, 1997). The frequency of chimera formation increases with:

- increased availability of partial rDNA fragments (Röling and Head, 2004).
- increased damage to DNA by restriction enzymes, UV irradiation, sonication, depurination and rigorous cell lysis (Röling and Head, 2004).
- increased percentage similarity between DNA templates (Wang and Wang, 1997).

The incidence of chimera formation can be decreased by:

- increasing the elongation time (Wang and Wang, 1997).
- decreasing the number of cycles and thereby limiting the opportunity for formation and amplification of chimeras (Röling and Head, 2004; Wintzingerode *et al.*, 1997).

Chimeras can also be identified after sequencing of amplified rDNA (Haas *et al.*, 2011). Chimeric sequences are difficult to distinguish from true biological sequences, however, there are several algorithms which search for and identify chimeric sequences. Chimeras can also be identified by the production of incongruent trees following phylogenetic analyses on opposite ends of the rDNA sequences (Röling and Head, 2004).

Differential amplification during PCR

As well as providing a measure of species diversity within a microbial community, 16S rRNA gene sequencing allows us to determine the relative abundance of species. This analysis relies on the assumption that all DNA molecules are amplified equally during PCR. However, whilst all rDNA sequences may appear equal, some are more equal than others. Differential amplification is a bias introduced by PCR which cannot always be corrected. Several factors have been identified as causing differential amplification of rDNA.

1. The rRNA gene copy number (*rrn* operon number) and genome size differ between species. Bacteria can have between 1 and 10 copies of the rRNA gene within their genome (Röling and Head, 2004). Additionally, the copy number of rRNA genes doesn't necessarily correspond to a regular increase in PCR product. Other factors such as density of rRNA genes and the percentage of the genome composed of rRNA genes have also been theorised to affect the efficiency of PCR amplification (Farrelly *et al.*, 1995; Stackebrandt *et al.*, 1999).

2. Intraspecific heterogeneity between rRNA genes exists. Not all the rRNA gene copies within a bacterial species will have the same sequence. By reviewing pairs of sequences from the same species in databases of rRNA gene sequences it has been estimated that up to 48% of sequence pairs have more variation than would be expected using a 1% random sequencing error. This variation is dependent on taxa so there is not an easy mathematical correction for this observation (Clayton *et al.*, 1995). Another study of heterogeneities found that 16S rRNA gene sequences from strains of *Paenibacillus polymyxa* differed from each other by one to eight nucleotides at ten loci in the V6

to V8 regions, without taking into account amplification errors (Nubel *et al.*, 1996). Intraspecific heterogeneity can complicate the quantification of bacteria and lead to an overestimation of diversity (Stackebrandt *et al.*, 1999; Röling and Head, 2004).

3. Differences in G+C content between sequences. rDNA sequences that have a lower G+C content denature with a higher efficiency and so may be preferentially amplified. This effect can be reduced by adding 5% acetamides, which also reduces the incidence of non-specific primer annealing (Reysenbach *et al.*, 1992).

4. Sequences outside the rRNA gene can inhibit amplification. Other DNA sequences and secondary structural features of the bacterial genome that serves as the original template DNA can inhibit PCR amplification of the rRNA gene. This is likely to be due to specific sequences as the use of different primers will differentially amplify the rRNA genes of different bacteria (Hansen *et al.*, 1998; Rainey *et al.*, 1994; Suzuki and Giovannoni, 1996). The effect cannot be overcome neither by using DNA denaturing cosolvents such as dimethyl sulfoxide and glycerol nor other techniques such as "touch down" PCR. It has been suggested that the effect can be minimised by using at least two different primer sets in separate amplifications then comparing the results (Hansen *et al.*, 1998).

5. Increasing template concentration reduces the rate of amplification. A critical concentration of template DNA exists at which reannealing of DNA strands is favoured over primer binding. When the concentration of template DNA reaches and supersedes this critical concentration, amplification is reduced. This allows other rDNA templates to be more effectively amplified in subsequent PCR cycles and will alter the relative abundance of rDNA sequences within the sample. This amplification bias is less likely to occur in samples with a wide variety of rDNA sequences at relatively low concentrations (Suzuki and Giovannoni, 1996).

6. Specificity of primers to the template DNA. Even if universal primers are used there is evidence to suggest that there is differential binding between primers and template

DNA from different bacterial species. Even single mismatches between primers and template DNA can reduce binding efficiency (Dahllöf, 2002). Suboptimal binding will result in decreased amplification of the respective template compared to others (Wintzingerode *et al.*, 1997). While lowering the annealing temperature will allow for mismatches it can also increase non-specific primer binding and unwanted products (Ishii and Fukui, 2001).

7. DNA contamination of PCR. Introduction of DNA to the sample can occur either through unintentional transfer of DNA from previous amplifications or by contamination of PCR reagents (Wintzingerode *et al.*, 1997). This is a particular problem for reagents such as DNA polymerase whose manufacture involves the use of *E. coli* (Röling and Head, 2004). To protect against this, a negative control must always be included which is handled the same as other samples except that no template DNA is added. Reagents should also be pre-treated with UV light or uracil DNA glycosylase to remove contaminating DNA (Niederhauser *et al.*, 1993).

Although PCR is an imperfect technique, it is currently the only reliable way of amplifying DNA from environmental samples. Nevertheless, it is important to take steps to minimise the introduction of biases. After amplification, the DNA from a sample can either be analysed directly using fingerprinting techniques such as DGGE, TGGE and T-RFLP or individual DNA fragments can be sequenced to identify the bacteria present.

1.2.4 Illumina sequencing

The Illumina MiSeq sequencing platform is commonly used for 16S rRNA gene amplicon sequencing and was the platform chosen for the experiments described in this thesis. Illumina MiSeq can sequence DNA from 96 samples and generate up to 25 million sequence reads per run. The first step in processing DNA samples for an Illumina MiSeq run is library preparation. DNA fragments in a sample are ligated with adapters and a 12 b.p barcode whose functions are to bind DNA fragments to a flow cell and to identify a fragment's sample of origin respectively. After library preparation, the adapter ligated

DNA is washed over a flow cell which is covered with oligonucleotides complementary to the adapter sequences. DNA fragments hybridise to these complementary sequences and are bound to the plate. Before sequencing can occur, each DNA fragment bound to the flow cell surface is replicated using bridge amplification to form a cluster of identical DNA fragments. Once clusters are formed, Illumina MiSeq employs a sequencing by synthesis technique to begin the sequencing process. This requires fluorescent tagged nucleotides with four colours representing each of the bases. After each round of nucleotide addition the nucleotides are stimulated to fluoress. An image taken of the flow cell after each nucleotide addition can be analysed to identify clusters and determine the sequence of the DNA fragment which composes each cluster. Illumina MiSeq also uses paired-end sequencing which produces sequence reads from both ends of a DNA fragment. Forward and reverse reads can be matched using an overlapping section during processing and produce a longer read length than would otherwise be possible (Illumina, Inc, 2017).

Studies assessing the reliability of Illumina MiSeq runs for 16S rRNA gene-based community profiling and whole genome assembly have been conducted. When compared to other next-generation sequencing platforms such as Ion Torrent PGM and Pacific Biosciences RS, Illumina MiSeq had fewer sequence errors, in one study producing error free reads in 75% of cases (Quail *et al.*, 2012; Loman *et al.*, 2012; Salipante *et al.*, 2014). Reproducibility of Illumina results between runs has also been assessed and found that separate runs from the same sample did not produce significantly different community profiles (Kennedy *et al.*, 2014).

1.3 Sequence Analysis

In the experiments presented in this thesis, the Quantitative Insights In Microbial Ecology version 2 (QIIME2) pipeline was used to analyse sequencing data. Comparisons between QIIME version 1 and other sequence analysis pipelines have shown that while different pipelines can produce differing results, especially for low abundance sequences and lower taxonomic levels, similar biological conclusions can be drawn from the analyses produced

(Allali *et al.*, 2017; López-García *et al.*, 2018; Plummer and Twin, 2016). As such, choice of sequence analysis pipeline is often down to personal preference, available training and prior knowledge. QIIME2 was chosen as the sequence analysis pipeline for these studies and is used consistently between them. Further discussion of sequence analysis will refer specifically to the methods employed in QIIME2.

1.3.1 Clustering sequences

A typical Illumina sequencing run will produce tens, if not hundreds, of thousands of sequences per sample. Now arises the problem of how those sequences can be grouped according to similarity so that relative abundance and other useful measures can be calculated. Previously, the basic unit of microbiome analysis was the "operational taxonomic unit" (OTU). This was defined as a collection of sequences with an assigned similarity, usually 97%, and was taken to be equivalent to a bacterial species. The benefit of the 97% threshold was that it avoided confusing sequencing errors with biological variation however biological variants are sacrificed in the process of avoiding sequencing errors (Eren *et al.*, 2016). With this loss of biological variation subtle ecological patterns at strain-level can be lost in the analysis (Berry *et al.*, 2017). Additional problems with the OTU method of clustering sequences come from the clustering methods themselves. Broadly, sequences can be assigned to OTUs using two methods: *de novo* OTU and closed-reference OTU clustering. *De novo* OTU assignment constructs clusters by comparing sequence similarity within the dataset. Various methods for constructing clusters have been used but all come with the same caveat: OTUs produced by *de novo* assignment cannot be compared outside of the dataset which produced them as the boundaries and assignment to a cluster are defined in relation to other sequences present within the dataset. *De novo* OTU assignment is a computationally costly method which excluded its use in large datasets. In contrast, closed-reference OTU clustering relies on comparing sequences in a dataset to a known reference database of OTUs. Sequences are assigned to known OTUs if sufficiently similar while sequences not matching the reference database are discarded. This process is computationally simpler but the quality of the results depends heavily on the quality

of the reference database. Samples from microbiomes with poorly defined membership could not be assigned OTUs using closed-reference clustering. A compromise method, open-reference OTU assignment, aimed to overcome this by first matching OTUs against a reference database then *de novo* clustering any unassigned sequences. However, the inability to subsequently compare datasets remains a stumbling block of the technique (Callahan *et al.*, 2017).

In recent years, the use of OTUs is being replaced by an alternative method for sequence clustering known as "amplicon sequence variants" (ASVs) which avoid clustering by an arbitrary threshold and instead cluster sequences based on 100% similarity. Where OTUs addressed confusing biological variation with sequencing error by assigning a threshold for similarity, techniques which produce ASVs attempt to remove sequencing error using mathematical models of patterns of expected error. This process is known as "denoising" and several tools, including DADA2, have been produced to denoise and organise sequences into ASVs (Callahan *et al.*, 2016). The use of ASVs has produced several benefits over OTUs including overcoming limitations of databases and allowing the comparison of results between studies. ASVs are consistent labels since the label is derived from the DNA sequence described. As a result, independently inferred ASVs can be directly compared between studies providing the same primers were used to produce the original sequences. In fact, this characteristic of ASVs makes the process computationally efficient. Previously, sequences from all samples would be pooled for *de novo* OTU assignment whereas ASV assignment is performed in samples individually allowing parallelisation of the process.

1.3.2 Taxonomy assignment

Once sequences have been sorted into ASVs the process of assigning taxonomy to each sequence begins. ASVs are compared to sequences in reference databases and assigned taxonomy by the degree of similarity between them. There are a variety of methods used to assign taxonomy to a sequence. The basic premise common to all methods is the use of an algorithm to compare an unknown sequence against a database of known sequences. This comparison informs the allocation of taxonomy to the lowest taxonomic level possible.

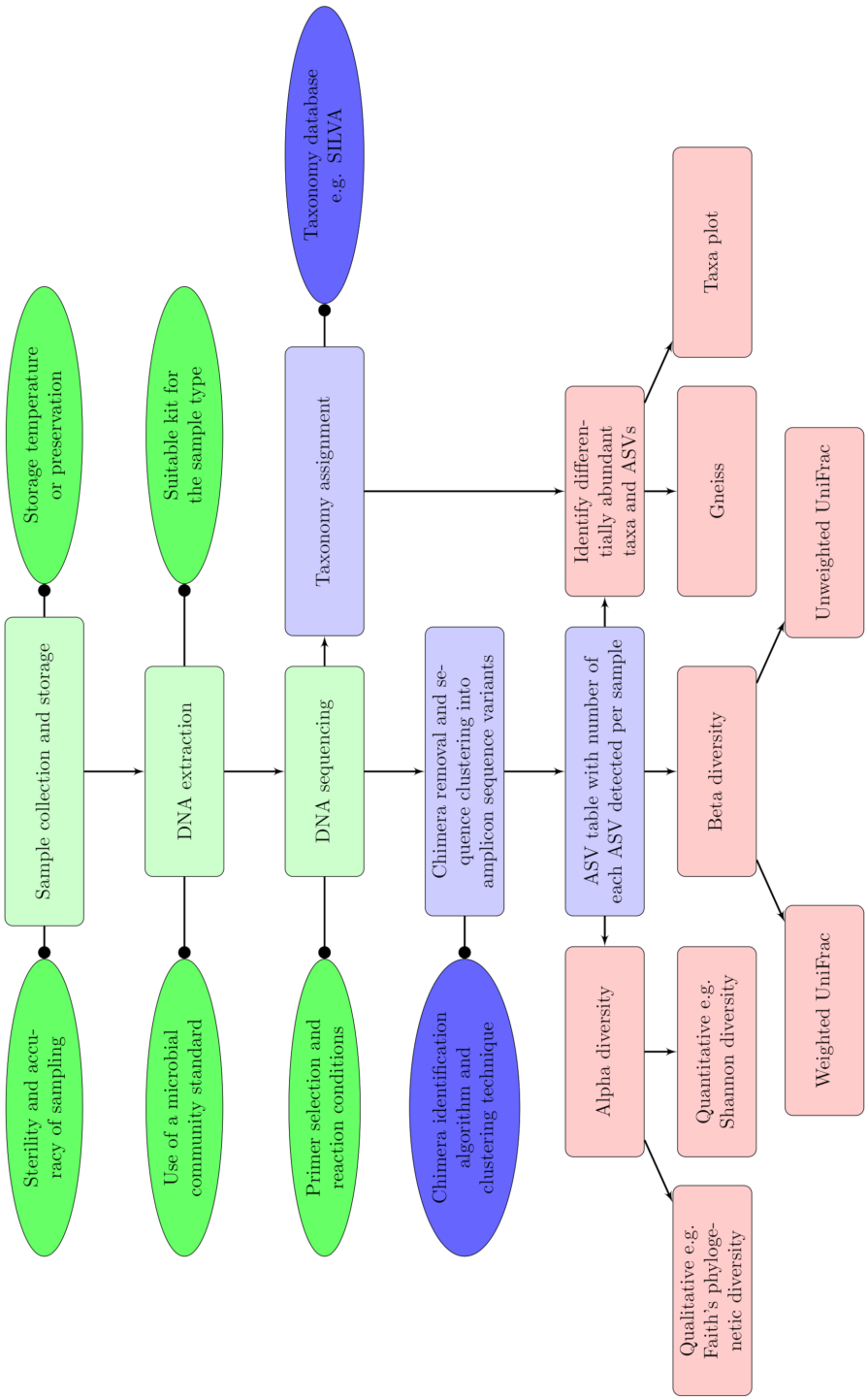


Figure 1.1: A pipeline for microbiome studies based on 16S rRNA gene amplicon sequencing

The flow chart describes the basic steps involved in determing microbiome composition of environmental samples from sample collection to analysis. Experimental stages (rectangles) can be divided into laboratory work (green), bioinformatic processing (blue) and analysis (red). Important considerations (ellipses) for laboratory work and bioinformatic processing are included.

Methods differing in either algorithm or reference database will produce slightly different results.

Sequence databases differ in size and curation method. The main databases used for taxnomy assignment in microbiome studies are SILVA (Yilmaz *et al.*, 2014), RDP (Brown *et al.*, 2013) and Greengenes (McDonald *et al.*, 2012). Of these, the SILVA database is the most up to date and contains the most reference sequences (Balvočiute and Huson, 2017), although it does have the disadvantage that the lowest taxonomic rank is genus, as opposed to Greengenes which assigns taxonomy to the species level. However, this disadvantage is largely negated when considering samples from poorly characterised communities with a high proportion of uncultured bacteria. Many sequences will not be identified to species or genus level, but instead will be assigned to the closest family. Additionally, accurate speciation of sequences will be hampered by the limited information provided by the 300 b.p read returned by Illumina sequencing.

In terms of the algorithm used to assign taxonomy to sequences based on the information in the database, QIIME2 taxonomy assignment is implemented by the q2-feature-classifier plugin (Bokulich *et al.*, 2018). As well as the option to use traditional alignment techniques implemented in BLAST+ and VSEARCH, the q2-feature-classifier plugin can use a multinomial naive Bayes machine-learning classifier available in the scikit-learn Python module (Pedregosa *et al.*, 2011) to assign sequence taxonomy. Although more computationally expensive, this QIIME2 method has been shown to produce accurate taxonomy assignment when compared to other pipelines and algorithms (Almeida *et al.*, 2018; Bokulich *et al.*, 2018).

1.3.3 Measuring diversity

Diversity is one of the key metrics by which microbial communities can be compared. Overall, diversity metrics fall into two broad categories depending on the aim of the analysis. Alpha diversity metrics aim to measure the diversity within a community while beta diversity aims to compare the diversity between two or more communities (Lozupone and Knight, 2008). Metrics can be further divided within alpha and beta diversity by whether they

are qualitative (taking into account only the absence or presence of ASVs) or quantitative (accounting for the abundance of each ASV).

Another consideration is whether to assume that ASVs are equally related (species-based measurement) or to take into account phylogenetic distance between ASVs (divergence-based measurements) (Lozupone and Knight, 2008). In their review of diversity metrics used in microbial ecology, Lozupone and Knight (2008) argue that divergence based methods are likely to be more useful in studies based on 16S rRNA gene sequences as bacterial species with closely matching 16S rRNA genes are often phenotypically similar.

One qualitative and one quantitative diversity metric were selected for alpha and beta diversities to be used in analysis of results.

Alpha diversity

Alpha diversity can be measured using a number of different metrics. Broadly, these metrics can be classified as qualitative, which disregards relative abundance, or quantitative which takes into account relative abundance. The differences between qualitative and quantitative metrics are summarised in Figure 1.2

Qualitative alpha diversity - Phylogenetic diversity

In terms of alpha diversity, qualitative metrics are equivalent to species richness and can be as simple as counting the number of ASVs identified in a sample. When methods such as clone libraries were more commonly used, species richness was estimated based on library coverage (Wise and Siragusa, 2007; Bjerrum *et al.*, 2006). Since next generation sequencing techniques provide adequate sequencing depth, estimations are redundant and a pure "Observed ASV" metric can be employed. However, this simple measurement does not take into account phylogenetic diversity. It would be hard to argue that a community composed of 100 species of Firmicutes contained the same diversity as one composed of 30 Bacteroidetes, 30 Firmicutes and 40 Proteobacteria. As such, Faith's Phylogenetic Diversity (FPD) measure (Faith, 1992) was selected as an appropriate qualitative alpha diversity metric. While FPD relies on the assumption that a community has been adequately sampled,

this should not be problematic providing an adequate sequencing depth has been achieved. However, since FPD relies on tree topology to calculate alpha diversity, results should not be compared between studies where different phylogenetic trees have been used to calculate FPD (Lozupone and Knight, 2008).

Quantitative alpha diversity - Shannon diversity index

Quantitative methods are synonymous with species evenness which takes into account the relative abundance of species within a community. Relative abundance of features within a community is an important factor to consider. Two communities with an identical taxonomic composition would be considered equally diverse when measured by FPD. However, it may be that in one community the relative abundance is dominated by a few ASVs whilst in the other there is a more equitable distribution of relative abundance between ASVs. A divergence-based quantitative metric has been described for use in microbial ecology (Martin, 2002) but it is not implemented in the current QIIME2 release. Shannon diversity is the most commonly used species-based alpha diversity metric (Reese and Dunn, 2018). The Shannon index increases with biological diversity and evenness. Another commonly used alpha diversity metric, Simpson's index, was rejected for use in this thesis. Simpson's index is designed to measure the probability that two randomly chosen members of a population belong to the same taxa or ASV (Simpson, 1949). Due to the distribution of relative abundance in microbiome data, in which one ASV may represent the majority of sequences with a multitude of lower abundance ASVs contributing the rest, Simpson's index often returns a value close to 1 in most communities. In contrast, the Shannon diversity index provides a balance between species evenness and richness resulting in its selection for use in this thesis.

Beta diversity

The benefits of using a divergence-based diversity metric have already been briefly outlined with respect to alpha diversity. A well established and robust beta diversity metric which can provide both qualitative and quantitative measurements is available in the form of the

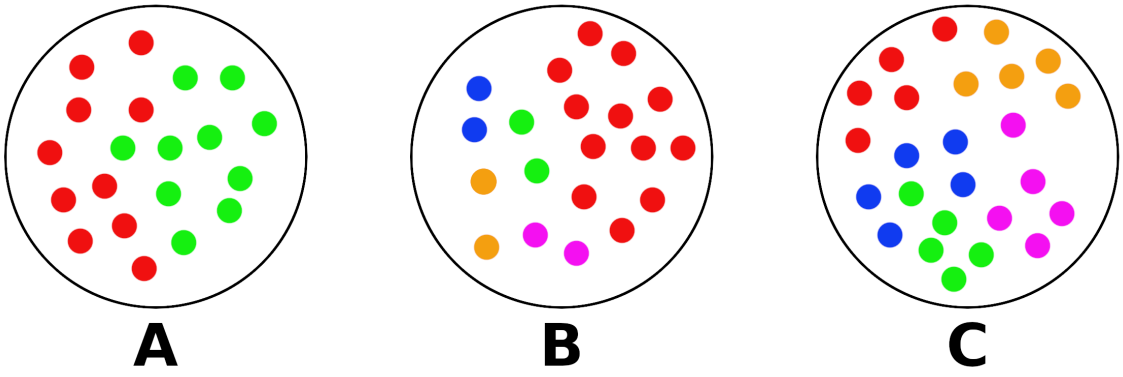


Figure 1.2: The differences between qualitative and quantitative alpha diversity metrics

In the example above, each community has 20 members with different colours representing different species. Community A will have the lowest alpha diversity regardless of whether a qualitative or quantitative metric is used. A qualitative metric will not be able to differentiate between Communities B and C as the number of species is the same. A quantitative metric will assign a higher diversity to Community C as relative abundance is not dominated by a single species as in Community B.

Unique Fraction (UniFrac) metric (Lozupone and Knight, 2005; Lozupone *et al.*, 2011). UniFrac measures the phylogenetic distance between communities by defining what fraction of the total branch length of a phylogenetic tree is occupied by taxonomic groups unique to one or other of the communities. This analysis can either be unweighted, giving a qualitative metric, or weighted, where branches of a tree are weighted according to the relative abundance of ASVs. It's beneficial to conduct both unweighted and weighted UniFrac analyses when comparing communities. For example, in a case where the ASV composition of communities is similar but the distribution of relative abundance is different, an unweighted UniFrac metric will not reveal any differences between communities whereas a weighted UniFrac will (Lozupone *et al.*, 2007).

A criticism leveled at UniFrac metrics is their tendency to favour detecting changes in either rare ASVs, in the case of unweighted UniFrac, or highly abundant ASVs in the case of weighted UniFrac. These biases may leave changes in moderately abundant ASVs unidentified. A generalised UniFrac metric has been proposed as a compromise which more accurately detects such changes, however, this metric has not been widely used in microbiome research (Chen *et al.*, 2012).

1.3.4 Identifying differentially abundant taxa

While alpha and beta diversity metrics can indicate that differences between communities exist they are not able to identify which ASVs are responsible for those differences. An initial method often used to observe and display the relative abundance of ASVs is a taxa bar plot. This allows a visual representation of the constituent parts of each community, collapsed at a single taxonomic level, allowing for some comparison. However, these relative abundances cannot be analysed as if they were absolute abundances. Unlike absolute abundances which are independent of one another, relative abundances display only the relationship between constituent parts. An increase or decrease in absolute abundance of any ASV within the community will result in a change in the relative abundance of all other constituent ASVs. In this sense, microbiome data can be defined as compositional and the tools used to identify differentially abundant ASVs must be suited to this data type (Gloor *et al.*, 2017).

Gneiss analysis was chosen to analyse differential abundance between groups since it overcomes challenges created by the compositional nature of high-throughput sequencing data. Firstly, a dendrogram of ASVs is prepared using correlation clustering. Each node in the dendrogram is treated as a "balance" with ASVs on one side of the balance termed numerators and on the other, denominators. Gneiss analysis examines the log ratio of abundances between numerator and denominator ASVs at each balance. Each log ratio's final numerical value is dependent on the balance between the ASVs composing the numerator and those composing the denominator of the ratio. Differences in the log ratio of a balance can be compared between sample groups to determine differences in microbiome composition. A significant difference between samples allows hypotheses to be formulated regarding changes in the absolute abundance of numerator and denominator ASVs but gives no further information as to which hypothesis is correct. For example, if balance y0 is found to be significantly lower at Time A compared to Time B the following hypotheses could explain the result:

1. The numerator ASVs have increased in abundance between Times A and B.

2. The denominator ASVs have decreased in abundance between Times A and B.

3. A combination of hypotheses 1 and 2.

4. Both numerator and denominator ASVs have increased in abundance between Times A and B, but numerator ASVs have increased more.

5. Both numerator and denominator ASVs have decreased in abundance between Times A and B, but denominator ASVs have decreased more.

Further investigations, such as quantitative PCR, are required to discern which hypothesis is correct (Morton *et al.*, 2017).

1.4 The Chicken Intestinal Microbiome

1.4.1 Composition of the chicken intestinal microbiome

Ultimately, the goal of modifying and controlling the microbiome for the benefit of the host must begin with an established normal or optimal state to aim towards. The composition of the intestinal microbiome can be influenced by many host factors such as genotype (Lumpkins *et al.*, 2010; Schokker *et al.*, 2015; Zhao *et al.*, 2013), as well as environmental factors from litter to diet (Cressman *et al.*, 2010; Park *et al.*, 2016). The microbial community also varies depending on the gastrointestinal organ sampled. Most work has focused on the caecum and jejunum; however, some information is available regarding the microbiome present in the upper gastrointestinal tract. An individual chicken's intestinal microbiome may be composed of between 250 and 350 different bacterial phylotypes, while around 640 bacterial species have so far been identified in the chicken gastrointestinal tract (Sergeant *et al.*, 2014; Apajalahti *et al.*, 2004). Comparison between studies is difficult due to differences in methodologies such as management, diet and chicken genotype. Moreover, Stanley *et al.* (2013*b*) demonstrated significant differences in intestinal microbiota between 3 trials despite the fact that the chickens were kept under the same conditions and fed the same diet. First exposure to microbes is critical in establishing a healthy intestinal

microbiota (Sergeant *et al.*, 2014). It is possible that the diversity between trials was caused by random exposure to environmental bacteria in the incubator and during transfer from the hatchery (Stanley *et al.*, 2013*b*). There is also a highly variable microbial diversity between individuals reared under the same conditions (Choi *et al.*, 2014; Sergeant *et al.*, 2014). Further variables are introduced during sample processing such as DNA extraction methods, PCR amplification and subsequent analysis. However, general patterns are apparent from examination of the literature, for example, the caeca are consistently identified as the most diverse gut compartment with a distinct microbiome to the small intestine, crop and gizzard (Bjerrum *et al.*, 2006; Choi *et al.*, 2014; Gong *et al.*, 2002*b*, 2007; Lu *et al.*, 2003; Van Der Wielen *et al.*, 2002). A summary of chicken gastrointestinal anatomy and the bacterial families most commonly identified by 16S rRNA gene sequencing in the foregut, midgut and hindgut is available in Figure 1.3.

Crop, gizzard and proventriculus

The crop is an enlarged section of the oesophagus present in most omnivorous and herbivorous bird species (Kierończyk *et al.*, 2016). The principal function of the crop in Galliformes is transitional digesta storage. The glandular stomach and gizzard have relatively low volumes so do not allow for constant feed intake. Once these organs are full, the crop acts as a store of feed for later digestion (Jackson and Duke, 1995). As well as storage, the crop acts to moisten feed and reduces the pH via bacterial fermentation but does not play an active role in enzymatic digestion (Kierończyk *et al.*, 2016). Crop pH can vary widely from 4.0 to 7.8 depending on a variety of factors including fill and subsequent bacterial activity (Kierończyk *et al.*, 2016). From the crop, food is passed to the proventriculus and gizzard which are homologous to the acid digestion stomach of mammals. The proventriculus secretes hydrochloric acid and pepsinogen for protein digestion while the gizzard provides for mixing and mechanical digestion (Svihus, 2014).

In terms of composition, the crop and gizzard have a similar microbiome which is distinct when compared to other gut compartments (Sekelja *et al.*, 2012). The dominant phylum present is invariably Firmicutes which forms between 40 and 60% of the community.

Proteobacteria is consistently the second most abundant phylum with a relative abundance between 10 and 20%, although this decreases with age (Choi *et al.*, 2014; Saxena *et al.*, 2016). *Lactobacillus* is the predominant genus found in the crop and gizzard (Choi *et al.*, 2014; Gong *et al.*, 2007; Sekelja *et al.*, 2012) with *L. reuteri*, *L. crispatus* and *L. salivarius* the most commonly isolated species (Abbas Hilmi *et al.*, 2007). One study, using sequences generated from a clone library, examined the microbial community at the species level. Gong *et al.* (2007) found that *L. aviaries* was the most abundant species found in the gizzard and *L. salivarius* was the most abundant in the crop. These species were less abundant in the small intestine and receive no mention in the discussion of caecal microflora. Although *Lactobacillus* forms the majority of the bacterial population, other taxa are present in the crop. Borda-Molina *et al.* (2016) compared the lumen and mucus microbiomes in the crop, finding that the crop mucus was further colonised by Lachnospiraceae, Burkholderiaceae, Ruminococcaceae and Streptococcaceae.

Small intestine

The small intestine is often divided into three anatomical sections, the duodenum, jejunum and ileum. The duodenum is defined as the first loop of the small intestine adjacent to the pancreas with the jejunum extending until Meckel's diverticulum and the ileum from there to the ileo-caeco-colic junction (Svihus, 2014). As in mammals, digesta is mixed with digestive enzymes and bile in the duodenum where fat digestion takes place (Sklan *et al.*, 1975). However, digesta is not detained for long in the duodenum with a relatively short retention time of approximately five minutes (Noy and Sklan, 1995). The majority of digestion and absorption occurs in the jejunum (Svihus, 2014). Despite its essential role in digestion and absorption, digesta is only retained in the jejunum for 40 to 60 minutes (Rougière and Carré, 2010; Weurding *et al.*, 2001). The function of the ileum is largely water and mineral absorption, however, there is evidence that the ileum plays a role in starch and fat digestion in fast-growing broiler chickens (Zimonja and Svihus, 2009; Svihus *et al.*, 2004; Hurwitz *et al.*, 1973).

Despite their differences in digestive function, there appears to be little difference in

microbiome composition between the three small intestinal compartments (Gong *et al.*, 2007). This allows the inclusion of results from studies where samples from small intestinal compartments were pooled or the authors did not specify which organ was sampled. As in the crop and gizzard, the small intestine is dominated by *Lactobacillus* with several studies identifying between 70 to 90% of sequences as belonging to this genus (Gong *et al.*, 2007; Choi *et al.*, 2014; Gong *et al.*, 2002*b*; Lu *et al.*, 2003; Witzig *et al.*, 2015). However, the most abundant species of *Lactobacillus* often differs between the crop and small intestine. For example, Witzig *et al.* (2015) found that *L. salivarius* was the most abundant species in the crop with a relative abundance of >46% while *L. crispatus* was the most abundant in the jejunum (>81%) and ileum (>77%). Early studies of the small intestinal microbiome suggested that other taxa were also present including Enterococcaceae, Streptococcaceae and Clostridiaceae (Gong *et al.*, 2002*b*; Lu *et al.*, 2003). With the advent of 16S rRNA gene sequencing and more complete databases for taxonomic assignment, a greater diversity of taxa have been identified in the small intestine. These have included Peptostreptococcaceae, Bifidobacteriaceae and Erysipelotrichaceae (Latorre *et al.*, 2018; Kollarcikova *et al.*, 2019). *Candidatus* Arthromitus is also increasingly identified within the early ileal microbiome and has been associated with increased performance although later colonisation with this taxa is associated with poor performance (Johnson *et al.*, 2018). There is also evidence to suggest that the mucus associated microbiome is different to the luminal microbiome with a lower relative abundance of Lactobacillaceae and a more diverse microbiome including Peptostreptococcaceae, Lachnospiraceae, Burkholderiaceae and Ruminococcaceae (Lu *et al.*, 2003; Borda-Molina *et al.*, 2016).

Caecum

Poultry have two caeca which comprise the last opportunity for digestion in the gastrointestinal tract. The caeca are blind-ending sacs whose main function is to provide a location for carbohydrate fermentation to take place. Most simple carbohydrates such as starch will have been digested and absorbed, however, plant material with a high proportion of dietary fibre are a major constituent of poultry diets. The carbohydrate components of dietary

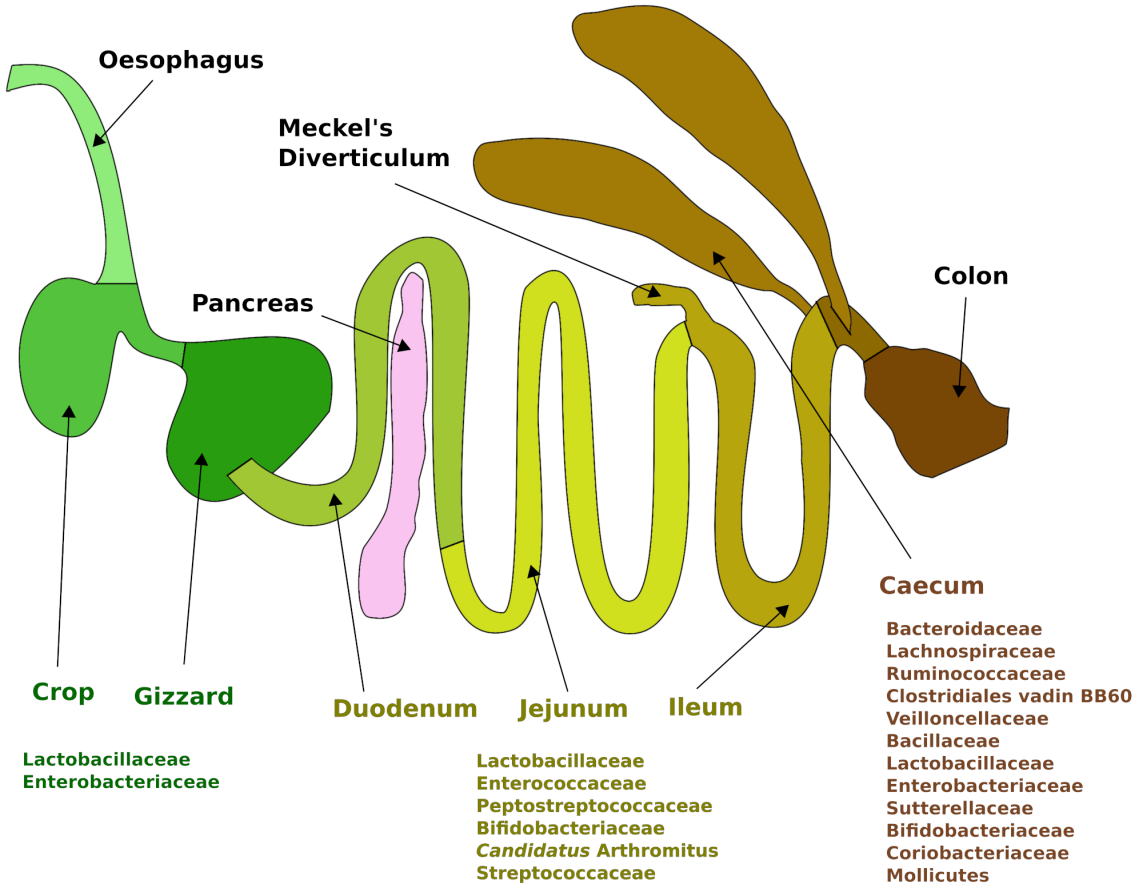


Figure 1.3: Anatomy of the chicken gastrointestinal tract and most commonly recovered bacterial families

The chicken gastrointestinal tract can be divided into three major anatomical divisions: the foregut, composed of crop and gizzard; the midgut, composed of duodenum, jejenum and ileum and the hindgut, composed of caecum, colon and cloaca. The diversity of the microbiome increases distally.

fibre, cellulose and hemicelluloses, are closely associated with non-carbohydrate compounds such as lignin, proteins and fatty acids (Józefiak *et al.*, 2004). This mixture of compounds provides a wealth of substrates which support a diverse bacterial community.

Analysis of the caecal microbiome shows a distinct and more diverse community to that present in other gut compartments. The dominant phylum varies between experiments with either Firmicutes or Bacteroidetes as the most abundant (Wei *et al.*, 2013; Lan *et al.*, 2002; Pandit *et al.*, 2018). Even though Bacteroidetes can be the most abundant phylum, the number of ASVs assigned to Firmicutes often outnumbers that of Bacteroidetes (Pandit *et al.*, 2018). Within Firmicutes, the families Lachnospiraceae and Ruminococcaceae form the bulk of relative abundance and are the most diverse (Sekelja *et al.*, 2012; Wei *et al.*, 2013; Gong *et al.*, 2007; Pandit *et al.*, 2018; Johnson *et al.*, 2018). Since these families are poorly characterised, many sequences are not assigned to genera, however, *Faecalibacterium*, *Blautia* and *Ruminococcus* are commonly found (Gong *et al.*, 2002*a*; Wei *et al.*, 2013; Johnson *et al.*, 2018; Ranjitkar *et al.*, 2016; Ijaz *et al.*, 2018). Other Firmicutes such as Lactobacillaceae, Clostridiales vadin BB60 group, Veilloncellaceae and Bacillaceae are found alongside Lachnospiraceae and Ruminococcaceae but at a lower relative abundance (Ijaz *et al.*, 2018; Johnson *et al.*, 2018; Ranjitkar *et al.*, 2016). In addition to families of Firmicutes and Bacteroidetes, other taxa such as Enterobacteriaceae, Sutterellaceae, Bifidobacteriaceae, Coriobacteriaceae, Mollicutes and Peptostreptococcaceae are often reported at lower abundances within the caecum (Wei *et al.*, 2013; Johnson *et al.*, 2018; Ijaz *et al.*, 2018; Pandit *et al.*, 2018). As with the crop and ileum there is some evidence that the mucus and lumen microbiomes differ, for example, Zhu and Joerger (2003) identified a higher number of Enterobacteriaceae in the caecal mucus when compared to the lumen although Borda-Molina *et al.* (2016) concluded that there was no difference in the relative abundance of the most common families.

Most of the current knowledge regarding composition of the chicken intestinal microbiome is derived from 16S rRNA gene sequencing studies. However, the limitations of this technique in terms of taxonomic classification and functional implications require researchers to use other techniques such as whole genome sequencing of bacterial cultures (Medvecky *et al.*,

2018). With recent advances in bioinformatics, whole bacterial genomes can be assembled from shotgun metagenomic data. This has revealed a new level of diversity within the chicken caecal microbiome with a recent study assembling genomes from 283 novel bacterial species (Glendinning *et al.*, 2020). The study by Glendinning *et al.* (2020) represents the only published study of metagenome-assembled genomes from the chicken microbiome. The taxonomy of these genomes largely reflects the features identified using 16S rRNA gene sequencing. A large proportion of the 469 draft genomes assembled were assigned to Firmicutes (n = 399) with the orders Oscillospirales (n = 179), Lachnospirales (n = 134) and Christensenellales (n = 17) the most abundant within this phylum. The family Ruminococcaceae is included within the order Oscillospirales (Glendinning *et al.*, 2020). Equivalent studies of the ileal microbiome have not yet been conducted.

1.4.2 Development and succession

Embryonic chicks

Before modern commercial systems were developed hens shared their microbiota with their progeny. Newly hatched chicks would quickly encounter a diversity of microbes from which they could derive their own intestinal microbiota. Modern hatcheries have created an automated environment to minimise disease transmission during the period of incubation, hatching and final delivery to farms. As a result, newly hatched chicks encounter a relatively clean environment and have no contact with the previous generation's microbiota (Pedroso *et al.*, 2016).

As well as acquiring new microbes from the environment, some authors have raised the possibility that chick embryos develop an intestinal microbiota *in ovo*. Viable bacteria have been identified in embryonic caecal tissue using FISH indicating that chicks may contain viable bacteria prior to hatching, however, no information is available concerning the proportion of positive to negative samples or identification of bacterial species (Pedroso *et al.*, 2016). Although the source of these microbes has not been definitively established some hypotheses have been suggested. Due to the anatomy of the hen reproductive tract it

is possible that the embryo is colonised by microorganisms before eggshell formation. These microbes subsequently enter the gut by ingestion of amniotic fluid during development or, if they are present in the yolk sac, internalised via the yolk (Pedroso *et al.*, 2016).

Day-old chicks

Day-old chicks sold by hatcheries are often defined as those younger than 72 hours old at the point of sale. Microbes present within the gut at this early stage are likely derived from the environment during incubation, hatching and handling during delivery. This initial colonisation is variable and the composition of the pioneer intestinal microbiome will differ significantly between chicks from different hatcheries (Pedroso *et al.*, 2005). This could explain the wide variety of results obtained by different research groups when examining the intestinal microbiome in day-old chicks. In general, the initial microbiome is dominated by Enterobacteriaceae or Clostridiaceae, although there are reports of high abundance of other taxa such as Streptococcaceae and Enterococcaceae (Johnson *et al.*, 2018; Ballou *et al.*, 2016; Oakley *et al.*, 2014*a*; Jurburg *et al.*, 2019). At the genus level, the most commonly isolated Enterobacteriaceae are *Escherichia/Shigella* while Clostridiaceae are most often assigned to *Clostridium sensu stricto* 1. Both of these genera are known to contain potential pathogens such as *Escherichia coli* and *Clostridium perfringens*. These bacteria are likely to be environmental in origin derived either from hatchery equipment or workers. Although the possibility of maternal microbiota transfer via the reproductive tract has been claimed, a recent study detected a high abundance of *Pseudomonas* in samples from the embyronic gut and egg albumin with no presence of Enterobacteriaceae of Clostridiaceae (Lee *et al.*, 2019).

It has been suggested that genotype plays a role in the microbiota of day-old chicks. Schokker *et al.* (2015) found different dominant bacterial genera in the jejuna of day-old chicks from two broiler lines known to produce different immunological responses to bacterial infection. However, the origin of the chicks is not stated in the paper. As noted before, this diversity could be caused by obtaining chicks from different hatcheries.

Successional development of the small intestinal microbiome

Once chicks have arrived on the farm they are exposed to a wider microbial environment with bacteria ingested from deep litter systems, feed and water. Succession occurs rapidly and diversion of populations in different gut compartments occurs at a young age. The caecal and ileal microbiota begin to diverge from about 3 days post-hatch (d.p.h) (Lu *et al.*, 2003; Van Der Wielen *et al.*, 2002). Wise and Siragusa (2007) examined the bacterial community in the ileum and discovered that Enterobacteriaceae followed a trend of decline over time. This decline was associated with increased abundance of *Lactobacillus* which replaced Enterobacteriaceae as the dominant taxa by 14 d.p.h. Schokker *et al.* (2015) described a faster transition to a higher abundance of *Lactobacillus*. The day-old microbiome was dominated by Enterococcaceae with a high proportion of *Escherichia* sequences and a small number of *Lactobacillus* present. By 4 d.p.h, the balance had reversed with *Lactobacillus* accounting for up to 88% of sequences accompanied by a decline in Enterococcaceae and *Escherichia*. Aside from *Lactobacillus*, there was an increase in microbial diversity over time with *Streptococcus* and some Clostridia contributing small numbers of sequences (Schokker *et al.*, 2015). The succession of *Lactobacillus* species in the ileum has not been studied in detail. Johnson *et al.* (2018) reported seven species of *Lactobacillus* in the ileum of chickens between 0 and 42 d.p.h. Only two of these species, *L. salivarius* and *L. crispatus*, were present at 0 d.p.h, with four at 7 d.p.h and all seven at 21 d.p.h. *Candidatus* Arthromitus colonises the ileum between 7 and 14 d.p.h (Johnson *et al.*, 2018; Jurburg *et al.*, 2019) and has been reported as the prominent taxa in the ileal mucus although *Lactobacillus* reclaims dominance at later time points (Wang *et al.*, 2016). Other slow growing taxa such as *Romboutsia*, a member of Peptostreptococcaceae, begin to colonise the ileum from 10 to 21 d.p.h (Jurburg *et al.*, 2019).

Successional development of the caecal microbiome

Ballou *et al.* (2016) described the caecal microbiome of day-old chicks as dominated by Gammaproteobacteria, mainly Enterobacteriaceae, with a smaller population of *Entero-*

coccus. Few studies have observed the microbiome at less than 3 d.p.h with most studies choosing to sample at 0 and 7 d.p.h. For the first three days of life, the microbiome maintains a low diversity although the dominant taxa may shift, for example, Jurburg *et al.* (2019) described an increase in *Escherichia/Shigella* abundance between 1 and 2 d.p.h. Significant shifts in microbiome composition begin to be reported from 3 to 4 d.p.h. These are characterised by an increase in Firmicutes, such as Ruminococcaceae and Lachnospiraceae, although the exact taxa often differs between studies (Ballou *et al.*, 2016; Gong *et al.*, 2019; Jurburg *et al.*, 2019). This increase in relative abundance of Firmicutes is often accompanied by a decrease in that of the original pioneer bacteria. The timing of appearance of Bacteroidetes varies between studies with some finding early appearance by 3 d.p.h (Gong *et al.*, 2019) while others report later colonisation up to 14 or 21 d.p.h (Johnson *et al.*, 2018; Jurburg *et al.*, 2019; Wise and Siragusa, 2007) or even no appreciable colonisation (Ballou *et al.*, 2016; Oakley *et al.*, 2014*a*). From this initial influx of colonisers, the diversity of the caecal microbiota continues to increase with new taxa of mainly Bacteroidetes and Firmicutes joining the community. Later colonising Firmicutes tend to belong to Ruminococcaceae with the genus *Faecalibacterium* particularly associated with the caecal microbiome from 14 to 21 d.p.h (Donaldson *et al.*, 2017; Johnson *et al.*, 2018; Jurburg *et al.*, 2019).

1.4.3 The effect of probiotics on the intestinal microbiome

As well as defining the normal composition and development of the chicken intestinal microbiome, researchers have focused on attempting to alter the microbiome composition using probiotics. Probiotics are live microorganisms (most commonly bacteria or yeasts) which are included in feed due to their beneficial effects (Lee *et al.*, 2010). Most research has been directed at the effect of probiotics on production parameters such as average daily gain and feed conversion ratio (FCR). The effect of probiotics on the colonisation of the gastrointestinal tract by pathogens has also been investigated in some depth. However, there is limited detailed information about the effect of probiotics on the composition of the intestinal microbiome. The difficulty with such experiments is differentiating the effect of

probiotics from other variables, such as environmental bacterial exposure, which are likely to alter the microbiome. Since probiotics are often delivered in feed, experimental groups need to be housed in different spaces which will invariably affect the composition of the intestinal microbiome.

The most commonly used probiotics tend to be *Lactobacillus* although *Bacillus*, *Bifidobacterium* and *Enterococcus* are also reported. Some probiotic preparations have included the yeast *Saccharomyces cerevisiae*. Probiotics are generally reported to have a positive impact on the intestinal microbiome, however comparison of results is impossible since different species, combinations and doses are used. The dose is particularly important as probiotic dose dependent effects have been observed (Kim *et al.*, 2012). Few studies have been conducted to assess the impact of oral probiotic administration on the composition of the chicken intestinal microbiome. Baldwin *et al.* (2018) found that oral administration of three *Lactobacillus* species at hatch did not change the overall composition of the faecal and caecal microbiota. Some differences were noted in the abundance of individual taxa including a reduction in Enterobacteriaceae and increase in Bacteroidaceae in probiotic treated chicks at 14 and 28 d.p.h (Baldwin *et al.*, 2018). A reduction in Enterobacteriaceae and other coliforms has been a common feature of probiotic experiments that used other techniques, such as culture, to measure the effect of probiotic administration on the microbiome (Samli *et al.*, 2010; Song *et al.*, 2014). Indeed, exclusion of pathogens such as *Salmonella* has been a measure of success for many studies involving probiotics. Higgins *et al.* (2008) were able to isolate *Salmonella* from fewer infected chicks also inoculated with a mixture of 11 different *Lactobacillus* species. The effect was partially dose dependent with no effect observed when *Lactobacillus* was administered at lower concentrations. Additionally, no effect was observed when chicks were treated with supernatant from probiotic cultures or a killed probiotic suggesting that living cells are required (Higgins *et al.*, 2008). Similar reductions in levels of *Campylobacter* have been achieved through probiotic treatments (Willis and Reid, 2008; Ghareeb *et al.*, 2012). The exact mechanism of action by which probiotics act to reduce the abundance of pathogens has not been published although current theories centre around competitive exclusion, immunostimulatory effects and improved epithelial

barrier function (Ng *et al.*, 2009; Adhikari and Kim, 2017). Whether or not probiotic bacteria exert this effect directly or by promoting colonisation and growth of a more diverse intestinal microbiome is not clear. Although probiotics may be selected because of direct *in vitro* effects on pathogens (Kizerwetter-Świda and Binek, 2016) there is evidence that probiotics can improve microbiome diversity. Gao *et al.* (2017) demonstrated more rapid development of the faecal microbiome after supplementation with *L. plantarum* via the drinking water. This included an increased abundance and diversity of other *Lactobacillus* species (Gao *et al.*, 2017). A more diverse microbiome is associated with increased stability and, therefore, the ability to resist insults such as pathogens or environmental stresses (Coyte *et al.*, 2015).

The delivery method of the probiotic also plays a role in the success of interventions. Most probiotics are administered directly in the feed, however, there is some evidence that microencapsulation can improve the efficacy of probiotics when compared to unencapsulated probiotics (Zhang *et al.*, 2015). Microencapsulation improves the survival of probiotic bacteria during transit through the upper gastrointestinal tract which may not provide a favourable environment (Mattila-Sandholm *et al.*, 2002). Intracloacal administration has also been explored as a method of improving probiotic efficacy. There is no information about the effect that intracloacal probiotic administration has on the composition of the intestinal microbiome, however, some differences in pathogen challenge trials have been noted. Intracloacal administration of probiotic was shown to significantly reduce the carriage of *Campylobacter* and *Salmonella* Enteritidis when compared to oral administration (Arsi *et al.*, 2015; Hashemzadeh *et al.*, 2010). It has also been shown that intracloacal administration is effective at lower concentrations when compared to oral administration (Higgins *et al.*, 2008). Another postulated method of administration is *in ovo* injection, whereby the probiotic is injected into the amniotic fluid at day 18 of incubation (Hashemzadeh *et al.*, 2010; Pedroso *et al.*, 2016). Pedroso *et al.* (2016) found that *in ovo* inoculation of bacteria cultured from adult caecal contents transiently changed the composition of the caecal microbiota with a decreased number of Proteobacteria in 7 day old chicks and a lower Firmicutes to Bacteroidetes ratio at 30 days old when compared to uninoculated chicks. However,

inoculation of more than 3.3 x 10^5 viable bacterial cells reduced egg hatchability (Pedroso *et al.*, 2016).

1.5 Importance of the Microbiome in Chickens

The microbiome is not an innocuous bystander in terms of host biology. As well as helping to reduce pathogen load the microbiome plays an essential role in host nutrition and immune development, both of which can be exploited to improve production either through improved feed utilisation or reduced losses from disease (Yeoman *et al.*, 2012).

1.5.1 Host metabolism and nutrition

In terms of nutrition, the microbiome can be viewed as an extension of the host genome. Like most other animals, chickens lack the ability to produce enzymes such as glycoside hydrolase and polysaccharide lyase which are required for the digestion of complex polysaccharides often present in plant material (Yeoman *et al.*, 2012). This is particularly important in the caecum which is the main site for complex carbohydrate digestion although further evidence is emerging that small intestinal bacteria also play a role in aiding digestion.

The small intestine

As previously discussed, the small intestine is the main site of digestion and nutrient absorption in the gastrointestinal tract. As the most abundant genus within the small intestine, *Lactobacillus* has been a focus of attention when considering the metabolic function of the microbiome. Presence of *Lactobacillus* has been correlated with improved host feed efficiency (Yan *et al.*, 2017). Many members of *Lactobacillus* have adaptations allowing them to successfully colonise and survive within the small intestine such as a resistance to bile acids and production of antimicrobial agents which facilitate colonisation at the expense of other species (Drissi *et al.*, 2017). Although the host produces endogenous enzymes capable of digesting lipids and simple carbohydrates, the presence of *Lactobacillus* may further facilitate digestion and absorption of these nutrients. Strains of *Lactobacillus*

have been shown to produce a wealth of enzymes that are able to ferment a wide repertoire of carbohydrates from disaccharides to more complex polysaccharides that would otherwise evade host digestion (Drissi *et al.*, 2017). For example, one strain of *L. sakei* was associated with a higher body mass index in humans suggesting that this species' ability to produce monosaccharides from complex polysaccharides may increase energy availability to the host (Štšepetova *et al.*, 2011). As well as aiding in carbohydrate metabolism, *Lactobacillus* can have a positive impact on lipid digestion and promote weight gain by producing thiolases which are a key component of beta oxidation of fatty acids (Drissi *et al.*, 2014). However, the effect of *Lactobacillus* on lipid digestion is strain dependent as several strains have been shown to produce bile salt hydrolases (BSH), a group of enzymes that reduce lipid digestion efficiency by catalysing the conversion of conjugated bile salts to unconjugated bile salts (Wang *et al.*, 2012). Despite being a commensal, the presence of *Lactobacillus* should not be viewed as a benevolent altruist working only to supply their host with a ready supply of nutrients. *Lactobacillus* is unable to synthesise amino acids and so competes with the host for this resource (Apajalahti and Vienola, 2016). Since most amino acid digestion and absorption occurs in the proximal small intestine, the host can take measures to create unfavourable conditions for bacterial growth in these areas. These measures may include production of antibodies and bile salts although the latter may not perturb *Lactobacillus*. Consequently, the density of the microbiota is lower in the proximal small intestine compared to the ileum which may maximise host amino acid absorption (Apajalahti and Vienola, 2016). While *Lactobacillus*' use of amino acids may be undesirable in the proximal small intestine, an increased population of *Lactobacillus* in the distal small intestine may be beneficial. Even with an optimal diet some protein will escape host absorption and enter the distal gastrointestinal system. Free dietary amino acids in the caecum are liable to be fermented by commensals, undergoing putrefaction which produces toxic end-products (Apajalahti and Vienola, 2016). In this respect, a healthy population of amino acid absorbing bacteria in the distal small intestine would be beneficial. Even bacterial competition for readily absorbable monosaccharides such as glucose may ultimately benefit the host. *Lactobacillus* is able to ferment glucose to produce lactate and

short chain fatty acids (SCFAs) such as acetate (Drissi *et al.*, 2017). These lower the pH of intestinal contents which inhibits the growth of potentially harmful bacteria such as Enterobacteriaceae. Less is known about the function of other small intestinal commensals since they are difficult to culture under laboratory conditions. However, the advent of full genome sequencing has facilitated the investigation of their metabolic capabilities. Genomic studies of *Romboutsia ilealis*, a species of Peptostreptococcaceae isolated from the human intestinal tract, have revealed adaptations similar to *Lactobacillus* that enable the bacteria to live in the small intestine. These include the ability to produce BSH and utilise carbohydrates that are not host-digestible as well as host-derived carbohydrates such as L-fucose which are abundant in intesintal mucus (Gerritsen *et al.*, 2017).

The caecum

The contribution of the caecal microbiome to host metabolism and nutrition is better understood than that of the small intestinal microbiome. Metagenomic analyses have highlighted the importance of the caecal microbiota in carbohydrate digestion with genes encoding for enzymes associated with carbohydrate digestion and transportation consistently the most abundant (Yeoman *et al.*, 2012). One study of the caecal metagenome found sequences of over 200 enzymes capable of degrading non-starch polysaccharides (Sergeant *et al.*, 2014). Monosaccharides released from the breakdown of non-starch polysaccharides are not utilised directly by the host, but are fermented by bacteria to produce SCFAs (Oakley *et al.*, 2014*b*). The principal SCFAs produced by monosaccharide fermentation are acetate, proprionate and butyrate. Sergeant *et al.* (2014) found complete pathways for the production of all three of these SCFAs in the chicken caecal metagenome with most of these genes associated with Bacteroidetes and Firmicutes. The SCFA profile within the caecum is highly dependent on the composition of the microbiota with higher numbers of proprionate producing genes associated with Bacteroidetes while Firmicutes are more likely to have genes for production of acetate, butyrate and formic acid (Polansky *et al.*, 2015). A recent study of whole genome sequences from chicken caecal bacterial isolates confirmed this pattern with genes required for butyrate production more commonly found in Ruminococcaceae

while the fermentation pathways present in Bacteroidetes genomes favoured the production of proprionate (Medvecky *et al.*, 2018). The recent use of metagenome-assembled genomes to study the chicken caecal microbiome may allow the identification of further metabolic pathways which benefit the host (Glendinning *et al.*, 2020).

SCFA have an important role in host health and nutrition as they can be absorbed and used as an energy source. The total contribution of SCFAs to host energy requirements is species dependent with humans obtaining 5 to 15% of their total energy from SCFAs whilst ruminants are almost entirely dependent on SCFAs produced by the rumen microbiota (Rinttila and Apajalahti, 2013) although little information is available regarding an equivalent figure in chickens. Butyrate has become of particular interest as it is the principal energy source of caecal enterocytes and has been shown to regulate cell differentiation and mitosis in intestinal crypts (Rinttila and Apajalahti, 2013). The beneficial effects of butyrate are not limited to provision of energy to host cells. It has also been shown to maintain the integrity of the epithelial barrier by promoting tight junction formation (Grilli *et al.*, 2016; Peng *et al.*, 2009). There is some suggestion from *in vitro* tests that this positive effect on tight junctions may promote the healing of intestinal mucosal wounds (Ma *et al.*, 2012).

1.5.2 Immune development

Structure of the chicken immune system

Birds and mammals shared a common reptilian ancestor more than 200 million years ago. As a result, there are certain common features of the immune system such as thymic development of T cells. However, there are other features which make the avian immune system fundamentally different such as the lack of defined peripheral lymph nodes and the presence of the bursa of Fabricius. The bursa is the site of B cell differentiation and maturation, so fulfilling a role similar to mammalian bone marrow, and acts as a secondary lymphoid organ that initiates adaptive immune responses to sampled antigens (Davison, 2014). Hindgut bacteria and cloacally applied antigens are sampled via retroperistalsis

from the cloaca (Smith *et al.*, 2014*a*). Exposure to gut derived molecules is essential for normal development of the bursa. Post-hatch maturation and development of B cells relies either directly or indirectly on exposure to microflora derived antigens (Ratcliffe and Härtle, 2014).

Gastrointestinal-associated lymphoid tissue (GALT) encompasses any leukocyte found within the gut and is comprised of lymphoid cells distributed throughout the epithelium (intraepithelial lymphocytes, IEL) and lamina propria as well as more defined lymphoid structures such as the caecal tonsils (CT), Peyer's patches (PP) and Meckel's diverticulum (Smith *et al.*, 2014*a*). The hindgut contains relatively more lymphoid aggregates than the foregut (Befus *et al.*, 1980), although organised lymphoid tissue has been found at the junction of the oesophagus and proventriculus (Oláh *et al.*, 2003), as well as in the lamina propria of the proventriculus (Jeurissen *et al.*, 1994). The small intestine contains up to six PP although their anatomic distribution is not constant with the exception of the most prominent found between 5 and 10cms proximal to the ileocaecal junction (Befus *et al.*, 1980). The caecum contains the most studied lymphoid tissue, the paired CT located at the proximal end of each caecum (Kitagawa *et al.*, 1998, 2000). Other less well defined lymphoid aggregates are present throughout the small and large intestines with the highest density found in the apical caecum (Kitagawa *et al.*, 1998). The colon lacks well defined lymphoid structures although follicles have been described in the cloaca (Befus *et al.*, 1980) and around the bursal duct (Friedman *et al.*, 2003).

The basic structure of the defined lymphoid aggregates is similar to that of mammals. The epithelium covering these regions includes specialised microfold cells whose function is to sample gut antigens and present them to macrophages and dendritic cells in the underlying tissue (Kitagawa *et al.*, 2000). Nearby are follicles composed of B and T lymphocytes which can mount an adaptive immune response. Although the structure of an avian lymphoid follicle is similar to that of mammals, the avian cellular composition is different. The IEL of the small intestine are mainly TCR$\gamma\delta$+, TCR$\alpha\beta$+ and NK cells (Lillehoj, 1994), with B cells largely absent (Vervelde and Jeurissen, 1993; Lillehoj *et al.*, 2004). Most of the T cells are CD8+ although there are smaller populations of TCR$\alpha\beta$+ CD4+ and CD4+ CD8+ cells

present (Vervelde and Jeurissen, 1993; Lillehoj *et al.*, 2004). Of the CD8+ cells, the majority express CD8αα homodimers instead of CD8αβ heterodimers more commonly present on systemic CD8+ T cells (Imhof *et al.*, 2000). The immune cell community of the lamina propria is composed of granulocytes, macrophages, dendritic cells and lymphocytes (Smith *et al.*, 2014*a*). The B cell population of the lamina propria mainly produces polymeric IgA (Bienenstock *et al.*, 1973a), which is then secreted across the epithelium by the polymeric Ig receptor (Bienenstock *et al.*, 1973b). The composition of the CT and PP is very similar to that of the lamina propria (Smith *et al.*, 2014*a*). Chickens are also different to mammals in that two subfamilies of TCRαβ+ cells are expressed, TCRαβ1+ (TCR2) and TCRαβ2+ (TCR3). TCR2+ cells dominate in the IEL population and are also relatively abundant in the lamina propria. The role of TCR2+ cells is linked to the production of enteric IgA, as depletion of these cells leads to a decrease in intestinal IgA production but leaves systemic IgM and IgY production unaffected (Cihak *et al.*, 1991). TCRγδ+ are also more abundant in the IEL than other sites (Cooper *et al.*, 1991). Although their biological function is still unknown, they have been shown to have cytotoxic capabilities *in vitro* (Chen *et al.*, 1994) and may have an immunoregulatory role (Quere *et al.*, 1990).

1.5.3 Development of GALT

At hatch, the gastrointestinal system is still immature. The epithelium continues to develop and increase in complexity during the first 3 to 4 weeks post-hatch (Vervelde and Jeurissen, 1993; Lilburn and Loeffler, 2015). During this time, GALT also displays an increase in cellular complexity and size (Vervelde and Jeurissen, 1993; Befus *et al.*, 1980) and these changes are in part dependent on the colonisation of the gut by its microflora (Honjo *et al.*, 1993). Some lymphocytes are already present in GALT at hatch. By 4 d.p.h, there has been a large increase in CD3+ cells (corresponding to T and NK cells) in all parts of the intestine (Bar-Shira *et al.*, 2003). Studies tracking the export of B and T cells have shown that a first wave of TCRγδ+ cells arrives at 15 to 17 days of incubation (d.i), followed by a wave of TCRαβ1+, TCRαβ2+ and B cells at 18 to 20 d.i (Dunon *et al.*, 1997). Around the time of hatching and at 6 to 8 d.p.h, more waves of TCRγδ+ cells are exported from

the thymus to the GALT, along with more TCRαβ1+ and TCRαβ2+ cells at 2 to 4 and 9 to 11 d.p.h (Dunon *et al.*, 1997). The number of B cells also increases, initially in the small intestine at 4 d.p.h, followed by the CT and large intestine at 6 d.p.h (Bar-Shira *et al.*, 2003). GALT lymphocytes are functionally immature upon arrival but mature in two phases, the first during the first week of life and the second during the second week of life (Bar-Shira *et al.*, 2003). Changes also occur in the composition of lymphocytes. During the first 4 weeks after hatch, there is an increase of TCRγδ+ cells and expression of CD8α in the IEL (Lillehoj and Chung, 1992). TCRαβ1+ cells also increase in the lamina propria (Lillehoj and Chung, 1992). Through experiments using gnotobiotic chickens it's apparent that many of the changes in lymphocyte populations are a consequence of gut colonisation by microbes (Honjo *et al.*, 1993).

Since the adaptive immune system is immature at hatch, the innate system must compensate to protect the chick from microbial challenges. Maternal IgA provides protection during the first 2 weeks of life (Lammers *et al.*, 2010). Maternal IgA is preserved in the gut by preferential uptake and secretion into the mucus layer by goblet cells, so limiting loss via intestinal flushing. Gallinacin, antimicrobial peptides homologous to β-defensins in mammals, expression is increased during the first 3 d.p.h before decreasing following increases in IL-8 and IL-17 associated with immune system maturation (Crhanova *et al.*, 2011).

1.5.4 The effect of gut microflora on intestinal development

Experiments using germ-free (GF) animals have shown that the presence of microorganisms within the gut is essential for normal structural and cellular development. Grossly, GF animals have been shown to have smaller guts with thinner walls when compared to conventionally reared (CR) chickens (Coates *et al.*, 1955). Microscopically, the villi are shorter with a thinner epithelium although functionally there is an increase in absorption and efficiency (Cook and Bird, 1973). Introduction of microflora to the intestine reverses these abnormalities and stimulates an increase in gut weight, cell turnover and villus height (Abrams *et al.*, 1963). Since GF mammals have been shown to be more susceptible to enteric

infections (O'Hara and Shanahan, 2006), it is reasonable to suggest that the microbiota also influences immune development. Colonisation of the gut by microbes begins immediately post-hatch. The initial interaction between host and microflora induces mild inflammation with infiltration of macrophages and heterophils (Crhanova *et al.*, 2011). There is increased expression of IL-8 and IL-17, peaking at 4 d.p.h and declining until 10 d.p.h (Crhanova *et al.*, 2011). It has been suggested that these interleukins mark a switch from a Th1 to a Th17 immune response conferring a resistance to pathogens such as *Salmonella* Enteritidis (Crhanova *et al.*, 2011). As well as functional markers, the microbiota influences immune cell populations in the GALT. Most notably, the lymphoid tissue in the CT is greatly reduced in GF compared to CR chickens (Hegde *et al.*, 1982; Honjo *et al.*, 1993), as well as there being fewer germinal centres (GCs) and plasma cells at the ileocaecal junction (Thorbecke *et al.*, 1955). The GCs of the intestine have been identified as important centres for generating immune system diversity and antigen affinity maturation of B cell receptors (Mehr *et al.*, 2004). Although some antibody diversity is present at hatch and is antigen independent, full maturation requires antigen exposure to develop food tolerance or a fully competent response to pathogens (Dibner *et al.*, 2008). This is elegantly demonstrated by Mwangi *et al.* (2010) who studied TCRβ repertoire in the spleen and different anatomical locations in the gut of GF, gnotobiotic (colonised with *Bacillus* spp.) and CR chickens. In GF birds, the TCRβ repertoire of the gut was identical to that of the spleen due to a lack of bacterial antigen exposure. The TCRβ repertoire in the gut of gnotobiotic birds differed to that of the spleen but showed little regional variation. However, there were differences in TCRβ repertoire between the gut and spleen, but also between gut regions in CR chickens, showing that TCRβ repertoire is dependent on the complexity of the intestinal microflora (Mwangi *et al.*, 2010).

Antigens from food can also play a role in the development of the intestinal immune system, although there is some conflicting evidence as to the extent of impairment caused by withholding food. As well as providing antigen stimulation leading to increased differentiation of immune cells (Bar-Shira *et al.*, 2005), feeding also supplies nutrients needed for the growth and development of lymphoid tissues (Dibner *et al.*, 1998). Bar-Shira *et al.* (2005)

observed a delay in immune development in the hindgut and bursa of Fabricius characterised by delayed antibody responses, expression of IL-2 and reduced γδ-T and B cell colonisation when food and water were withheld from chicks for 72 hours post-hatch. These negative effects persisted for 2 weeks. Importantly, the chicks were not handled or removed from the incubator during these 72 hours to reduce exposure to bacteria (Bar-Shira *et al.*, 2005). In contrast, Simon *et al.* (2014) found no difference in cytokine or immunoglobulin expression between chicks fed immediately post-hatch and those starved for 72 hours. However, starved chicks were handled and placed in pens so it cannot be ruled out that exposure to microbes produced a cytokine and immunoglobulin response in the gut. There is evidence to suggest that the deleterious effects of food deprivation can be observed if feed is withheld for 24 hours and the impact of starvation increases with time post-hatch (Juul-Madsen *et al.*, 2004; Panda *et al.*, 2010; Bhanja *et al.*, 2009).

The complexity of the intestinal microflora has been shown to affect the composition and dynamics of intestinal mucin. The mucin layer covering the intestinal epithelium is formed of two sublayers: an outer, loose layer where bacteria are able to colonise and an inner, compact layer which prevents bacteria from binding to and invading the epithelium (Hansson and Johansson, 2010). Mucins can be classified into three groups based on histochemical staining: neutral, sialic acid-containing and ester sulphate-containing mucins (Filipe, 1979). The functional significance of these groups has not yet been defined. The importance of the mucin layer has been highlighted by research showing that differences in the pathogenicity of *Campylobacter jejuni* between humans and chickens is caused by the attenuating effect of chicken intestinal mucus (Byrne *et al.*, 2007). Smirnov *et al.* (2005) showed that chickens whose intestinal microbiome has increased *Bifidobacterium* or *Lactobacillus*, because of antibiotic growth promotor or probiotic use, displayed increased expression of mucin mRNA in the small intestine. This didn't result in an increased thickness of the adherent mucus layer leading to the conclusion that turnover had increased. An increased mucus turnover would be beneficial in terms of preventing penetration of the inner mucus layer by bacteria (Smirnov *et al.*, 2005). In terms of composition, mucin from the jejunum of CR chickens has a higher level of sialylated than sulphated mucin, while that of low bacterial load

chickens is primarily composed of sulphated mucin (Forder *et al.*, 2007). These results have been mirrored in studies with GF chickens, in which it was shown that GF chickens have increased sulphated mucin, absence of sialylated mucin and decreased mucin mRNA expression in the small intestine when compared to CR chickens (Cheled-Shoval *et al.*, 2014).

1.5.5 Competitive exclusion of pathogens

The role of the microbiota in excluding potential pathogens from the gastrointestinal tract was first demonstrated in the early 1970s. Rantala and Nurmi (1973) described how orally gavaging newly hatched chicks with intestinal contents from healthy adults reduced *Salmonella* colonisation and termed the effect competitive exclusion (CE). Soon after, commercial mixtures of bacteria such as Broilact™ and Aviguard™ became available. These were undefined mixtures of bacteria cultured from the caeca of healthy adults. However, as DNA sequencing technology has revealed, our inability to successfully culture the majority of chicken caecal bacteria will have resulted in a mixture not entirely unlike the original caecal microbiome composition. Despite this shortcoming, Broilact™ has been shown to reduce *Salmonella* colonisation in treated chicks (Wierup, 1988; Nuotio *et al.*, 1992). While factors such as the microbiota's role in intestinal immune development and production of SCFAs will contribute to the success of CE treatments, as previously discussed, further mechanisms for the action of CE mixtures have been proposed.

It has long been known that bacteria can produce factors that inhibit the growth and colonisation of other species. The members of the intestinal microbiome are no exception. Some of these factors may be metabolites such as SCFAs or hydrogen peroxide whereas others are released with the purpose of causing harm to other bacteria (Tiwari *et al.*, 2012). One such class of molecules are bacteriocins. Several genera of bacteria, including *Lactobacillus* and *Enterococcus*, have been shown to produce bacteriocins with the ability to inhibit a range of common pathogens from *Campylobacter jejuni* to *Clostridium perfringens* (Pan and Yu, 2014). If pathogenic bacteria do become established in the lumen, they often need to adhere to the epithelium before causing disease. The commensal microbiota which

normally occupy the mucus layer represent a further barrier which pathogens must overcome. Commensals may exclude pathogens from the mucus layer by occupying potential binding sites, stimulating mucus production and even producing factors which inhibit invasion of epithelial cells (Oelschlaeger, 2010). As well as competing for binding sites, commensal bacteria will compete with pathogens for limited resources.

1.6 Aims of This Study

In light of the important role that the intestinal microbiome plays in growth, development and disease resistance in chickens; further work to define the normal development of the ileal and caecal microbiome is required (Chapter 2). Once a normal pattern of succession without interventions has been defined, the success of interventions can be measured against this in terms of speed and adequacy of colonisation. The first involved investigating the effect on microbiome development in the ileum and the caecum of applying diluted adult caecal contents to the egg surface during incubation. The aim was to observe the effect of this intervention on the time of first colonisation and relative abundance of important taxa identified in the first experiment. Ileal and caecal morphology were examined as parameters of intestinal development to determine whether there was a correlation between the composition of the microbiome and faster intestinal development and colonisation by immune cells (Chapter 3). As a secondary aim, the study allowed identification of bacterial taxa which are amenable to transfer to chicks via topical application of caecal contents to eggs during incubation. The second intervention was an oral probiotic delivered via the feed. The primary outcome was to measure the influence of a single taxon on development of the ileal and caecal microbiome (Chapter 4). The aim of this experiment was to compare the effect of the oral probiotic on intestinal microbiome development with reference to the results of both the first and second experiments to determine any alteration from normal development and to identify which of the two interventions had a greater effect.

Chapter 2

The Development of the Chicken Intestinal Microbiome

2.1 Introduction

The intestinal microbiome represents a diverse bacterial community often containing between 200 and 350 different bacterial species (Sergeant *et al.*, 2014). In recent years it has become apparent that this diverse range of bacteria are not innocuous bystanders but play a range of roles in the host, from metabolism to immune maturation (Oakley *et al.*, 2014*a*). Advances in the field have spurred efforts to identify beneficial bacteria and modulate their abundance to accentuate their effects. However, before successful interventions can be implemented a more detailed understanding of the normal development of the intestinal microbiome is required. In the face of antibiotic resistance and a need to find new approaches to infectious disease control, the question of how certain bacteria modulate host immunity is of particular interest to the broiler industry. While some bacteria have been identified as potential candidates for immunomodulatory probiotics there are still gaps in our knowledge regarding the normal development of the ileal and caecal microbiota in chickens.

Previous work describing bacterial succession in the caecum and ileum has been discussed in Chapter 1.4. Observational studies of the intestinal microbiome are limited. Additionally, most of these studies were conducted using techniques such as denaturing gradient gel electrophoresis and clone libraries which have been superseded by next generation sequencing (NGS) technology. More recent studies which have used NGS focus on differences in microbiota composition between a treatment group and an untreated control. Consequently, there is a lack of current literature observing the normal development of the intestinal

microbiome using the increased resolution of NGS and advances in bioinformatics such as denoising ASV assignment and updated taxonomic databases.

There is also some evidence that the intestinal microbiome can be influenced by host genotype in chickens (Schokker *et al.*, 2015; Lumpkins *et al.*, 2010; Zhao *et al.*, 2013). These studies have used divergent or inbred lines rather than commercial breeds making the results of limited direct use to the poultry industry. Another potential source of variation is that the experimental groups are often housed separately. Since environmental factors such as litter type and diet (Cressman *et al.*, 2010; Park *et al.*, 2016) are known to affect the composition of the microbiome it is reasonable to question whether housing experimental groups separately introduces a confounding variable.

This study aimed to revisit the topic of normal ileal and caecal microbiome development using the increased resolution of NGS to shed light on microbial succession. The second objective of this study was to observe the development of the caecal and ileal microbiomes in three common breeds of broiler chicken (Cobb 500, Hubbard JA87 and Ross 308) whilst they are housed together. This data was intended to act as a baseline measurement for further experiments which sought to alter the composition of the intestinal microbiome using oral probiotics and topical application of caecal contents from healthy chickens to incubating eggs.

2.2 Materials and Methods

2.2.1 Animals and housing

One hundred and ten (36 Cobb 500, 38 Hubbard JA87 and 36 Ross 308) 'day-old' chicks were obtained from the same commercial hatchery. Chicks were distributed across three circular brooder pens (2 metres diameter) in the same room of a climate-controlled, biosecure chicken housing unit. Each brooder used a wood shaving substrate and contained the same number of chicks from each breed. Chicks were tagged with coloured wing tags to allow accurate identification of the different breeds but were not individually identifiable. Water and feed were provided *ad libitum* by a drinker and feeder in each brooder. Chicks were

fed a pelleted vegetable protein-based starter diet (Special Diet Services, Witham, Essex, UK) until 14 days post hatch (d.p.h). From 14 d.p.h a pelleted vegetable protein-based grower diet (Special Diet Services, Witham, Essex, UK) was provided until the end of the experiment. Nutritional composition of the starter and grower diets is displayed in Table 2.1 with a full list of ingredients in Table A.1. No coccidiostats or antimicrobials were added to either diet due to the high biosecurity levels maintained in the housing. At 22 d.p.h the birds no longer required brooder lamps and, as such, they were removed from the brooders and housed together in the same room on wood shavings. Temperature in the birds' pens was maintained between 25 and 30°C. No mortality was observed during the study. All experimental protocols were conducted in accordance with the Animals (Scientific Procedures) Act 1986 under Project Licence 40/3652 and was approved by the University of Liverpool Animal Welfare and Ethical Review Body prior to the award of the licence.

Analytical Constituents (%)	**Diet**	
	Starter	**Grower**
Crude Fat	2.7	2.4
Crude Protein	18.9	15.6
Crude Fibre	3.8	4.1
Crude Ash	6.6	5.6
Lysine	0.99	0.69
Methionine	0.44	0.27
Calcium	1.05	0.89
Phosphorus	0.7	0.62
Sodium	0.15	0.15
Magnesium	0.17	0.22
Copper	15 mg/kg	16 mg/kg

Table 2.1: Nutritional composition of starter and grower diets

2.2.2 Sample collection

Samples were taken from 5 chickens of each breed at 0, 3, 7, 14, 21, 28 and 42 d.p.h. After euthanasia by cervical dislocation, the abdomen was sprayed with 70% ethyl alcohol. Skin

incisions were made to expose the sternum which was then reflected to give good access to the coelom. The caecum and ileum were removed carefully to avoid external contamination. The content of one caecum and a 5cm section of ileum were manually expressed into sterile containers. Any visible digesta remaining in the caecum and ileal section was manually expressed. The caecum and ileum were opened longitudinally using a sterile scalpel which was then used to gently scrape off the mucus layer and transfer it to a sterile container. It was not possible to take all samples at every time point. At 0 d.p.h, only caecal content was taken due to a lack of digesta in the ileum. Mucus samples were taken from 14 d.p.h as the intestines were too small to yield enough mucus for accurate pooling.

At 3 and 7 d.p.h, caecal samples were weighed, diluted with 500µl of sterile water, pooled according to breed and homogenised. Ileal samples from 3 d.p.h were weighed and pooled since some chicks yielded less than 200mg of content. For all other time points, 200mg of luminal content from each bird was taken and pooled according to breed. Mucus samples were weighed, diluted with 500µl of sterile water, pooled according to breed and homogenised. The pooled samples were flash frozen in liquid nitrogen and stored at -20°C for 5 weeks before DNA extraction.

Caecal mucus samples from the fifteen birds sampled at 42 d.p.h were stored for individual sequencing to allow an insight into how representative pooled samples are of their constituent samples. In these cases, 500µl of sterile water was added to each sample of which 250µl was pooled and the remaining 250µl stored for individual DNA extraction.

2.2.3 DNA extraction

DNA was extracted from each sample using Zymobiomics DNA MiniKits (Cambridge Bioscience, UK) according to the manufacturer's instructions. DNA was extracted from 200mg of luminal samples and 250µl of homogenised mucus. An initial bead-beating step was performed using a Qiagen TissueLyser at 30Hz for 10 minutes. DNA was extracted from samples serially to ensure that storage time was equal for each sample point. At each extraction, two controls were included: a blank extraction to control for contamination and 75µl of Zymobiomics Standard Bacterial Community (Cambridge Bioscience, UK) to

control for variations in DNA extraction efficacy.

Extracted DNA was quantified using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies) and a Qubit dsDNA HS fluorometric kit (Invitrogen).

A trial PCR was also conducted to confirm the presence of bacterial 16S rRNA genes and suitability for MiSeq library preparation. Primers used spanned the V4 region of the 16S rRNA gene (F: 5'-TGCCAGCMGCCGCGGTAA-3', R: 5'-GGACTACHVGGGTWTCTAAT-3', (Caporaso *et al.*, 2012)).

2.2.4 Illumina MiSeq sequencing

Extracted DNA was sent for paired-end sequencing of the 16S rRNA gene at the Centre for Genomic Research (University of Liverpool) using an Illumina MiSeq run. The V4 hypervariable region (515F/R806) was amplified to yield an amplicon of 254 base pairs (Caporaso *et al.*, 2011). Library preparation was performed using a universal tailed tag design with subsequent amplification performed using a two step PCR with a HiFi Hot Start polymerase (Kapa) (D'Amore *et al.*, 2016). The first round of PCR was performed using the primers 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGTGCCAGCMGCCGCGGTAA-3' (forward) and 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT-3' (reverse) (D'Amore *et al.*, 2016). The raw Fastq files were trimmed for the presence of Illumina adapter sequences using Cutadapt version 1.2.1. The reads were further trimmed using Sickle version 1.200 with a minimum window quality score of 20. Reads shorter than 10 base pairs after trimming were removed. Raw sequence reads are available in the NCBI Sequence Repository Archive under the accession number SRP158778.

2.2.5 Amplicon sequence variant identification and taxonomy assignment

QIIME2 version 2019.1.0 was used for analysis of the Illumina data (Bolyen *et al.*, 2019). Amplicon sequence variant (ASV) assignment was completed using the dada2 plugin (Callahan *et al.*, 2017) and a feature table produced in biological observed matrix format

(McDonald *et al.*, 2012). Taxonomy was assigned using the q2-feature-classifier plugin (Bokulich *et al.*, 2018) with a pre-trained NaiveBayes classifier based on the SILVA 132 database of the 515F/R806 region of the 16S rRNA gene (Yilmaz *et al.*, 2014) available for download at https://docs.qiime2.org/2018.11/data-resources/.

2.2.6 Data analysis and statistics

Samples were divided into seven groups for analysis depending on the experimental question (Table 2.2). Alpha and beta diversity analyses were performed at a sampling depth of no less than 16,000 (depending on the sample group analysed) using the alignment (Katoh and Standley, 2013), phylogeny (Price *et al.*, 2010) and diversity (https://github.com/qiime2/q2-diversity) plugins. Alpha diversity was measured using an observed ASVs metric and Faith's phylogenetic diversity (FPD) metric to measure species richness and a Shannon diversity (SD) index to measure species evenness (Chapter 1.3.3). These metrics were compared between sample groups using a Kruskal Wallis test with a false discovery rate (FDR) correction. Taxa plots were produced using the q2-taxa plugin (https://github.com/qiime2/q2-taxa). Beta diversity was calculated with an unweighted and a weighted UniFrac metric. The beta diversity matrix was used to draw principal coordinates analysis (PCoA) plots and an ANOSIM test was used to determine the significance of differences in beta diversity between sample groups.

Gneiss analysis (Morton *et al.*, 2017) was chosen to analyse differential abundance between groups since it overcomes challenges created by the compositional nature of data derived from NGS of environmental 16S rRNA gene profiles (Chapter 1.3.4). To facilitate interpretation of results, less common ASVs were filtered by excluding ASVs with a total frequency less than the median total frequency. Gneiss analysis was run using the gneiss plugin (https://biocore.github.io/gneiss/) to identify taxa which were differentially abundant between time points, area sampled and breeds. Principal balances for use in Gneiss were obtained via Ward's hierarchical clustering using the correlation-clustering command. Isometric log ratios for each balance were calculated using the ilr-transform command. A multivariate response linear regression model of log ratio balances was constructed with

'Area' , 'Breed' and 'Days post-hatch' as covariates using the ols-regression command. Results were visualised through a regression summary and dendrogram heatmaps. Balances significantly affected by the covariates 'Days post-hatch' , 'Breed' and 'Area' were identified as those with a p-value less than 0.05. The results of this analysis were used to select taxa for further analysis using quantitative PCR.

Group	**Samples Included**	**Analysis**
All Samples	Caecal and Ileal, Lumen and Mucus, 0 - 42 d.p.h	General patterns and differences between caecal and ileal samples
Pooled Caecum	Caecal, Lumen and Mucus, 0 - 42 d.p.h	Succession in the caecal microbiome
Pooled Ileum	Ileal, Lumen and Mucus, 3 - 42 d.p.h	Succession in the ileal microbiome
Individual vs Pooled	Caecal, Mucus, 42 d.p.h	Differences between breeds, Validation of pooling method
Lumen vs Mucosa: Caecum	Caecal, Lumen and Mucus, 21 - 42 d.p.h	Differences between luminal and mucosal microbiomes in the caecum
Lumen vs Mucosa: Ileum	Ileal, Lumen and Mucus, 14 - 42 d.p.h	Differences between luminal and mucosal microbiomes in the ileum
Standards	Community and DNA Standards	Validation of extraction and sequence analysis

Table 2.2: Sample groups used for analysis

2.2.7 Quantitative PCR

Taxa were selected for further exploration using quantitative PCR based on results from Gneiss analysis. A literature search was conducted to find suitable primers. Where suitable primers were not available, the sequences retrieved from Illumina sequencing were used to produce taxa specific primers. The sequence was input into Primer-BLAST and a suitable

primer pair was chosen. To test specificity of primers, each primer pair was input into TestPrime for comparison against the SILVA database SSU-r132. Further testing of primers was conducted using PCR. The primers were tested against known positive and negative samples to check for the correct amplicon size and non-specific amplification. A gradient PCR was conducted to establish the correct annealing temperature for quantitative PCR. Primers used are displayed in Table 2.3.

The real-time quantitative PCR assay was conducted on a 1:10 solution of extracted DNA with a Rotor-Gene Q cycler (Qiagen) and Rotor-Gene SYBR Green PCR kits (Qiagen). The V4 region of the 16S rRNA gene was used as a reference gene. Rotor-Gene Q software (version 2.3.1.49) was used to produce melting curves and identify the cycle threshold (Ct), the point at which fluoresence above the background level is detectable. Each sample was run in triplicate with an averaged Ct used in further analysis. The ΔCt, defined as the difference between the Ct value for taxa specific primers and the Ct value for the reference gene, was calculated for each sample. Results were expressed as 40 - ΔCt. Results from qPCR were compared to the relative abundance of the corresponding taxonomic group using a Spearman rank-order correlation coefficient to assess correlation between abundance determined by qPCR and sequencing.

Target Taxa	**Primers**	**Amplicon Size (b.p.)**	**Reference**
Domain Bacteria (targets V4 region)	F: TGCCAGCMGCCGCGGTAA R: GGACTACHVGGGTWTCTAAT	254	(Caporaso *et al.*, 2012)
Bacteroides	F: CCTWCGATGGATAGGGGTT R: CACGCTACTTGGCTGGTTCAG	131	(Tanaka *et al.*, 2016)
Lachnospiraceae-Ruminococcaceae	F: CGGTACCTGACTAAGAAGC R: AGTTTYATTCTTGCGAACG	429	(Nava *et al.*, 2011)
Bacillus	F: GCATTGGAAACTGGGGGACT R: CCGGTGTTCCTCCACATCTC	90	This study
Bifidobacterium	F: CTCCTGGAAACGGGTGG R: GGTGTTCTTCCCGATATCTACA	550	(Matsuki *et al.*, 2004)
Clostridium cluster IV	F: TTACTGGGTGTAAAGGG R: TAGAGTGCTCTTGCGTA	580	(Van Dyke and McCarthy, 2002)
Clostridium cluster XIV a&b	F: AAATGACGGTACCTGACTAA R: CTTTGAGTTTCATTCTTGCGAA	438-441	(Matsuki *et al.*, 2002)

Table 2.3: Primer pairs used for quantitative PCR of bacterial taxa

2.3 Validation of Methodology

2.3.1 Analysis of community standards

Throughout the three experiments presented in this thesis, two standard communities were used as controls: a standard community from which DNA was extracted (Zymobiomics Microbial Community Standard, Cambridge Bioscience, UK) and a DNA standard community (Zymobiomics Microbial Community DNA Standard, Cambridge Bioscience, UK) which were submitted for sequencing. The composition of each of these standards is displayed in Table 2.4. A sampling depth of 42,000 was used for diversity metrics. An observed ASV metric was used to measure alpha diversity rather than FPD since the number of ASVs was deemed to give a better assessment of possible contamination.

Alpha diversity

There were no significant differences in alpha diversity between Community and DNA standards. Community standards had a mean of 14 ASVs with a range of 11 to 32 ASVs. DNA standards had a mean alpha diversity of 13 ASVs and a range of 11 to 20 ASVs. There were significant differences in alpha diversity between Community standards used in the experiments presented in Chapter 2 (mean ASVs = 13.9; Std Dev = 1.1) and Chapter 3 (mean ASVs = 16.3; Std Dev = 1.5) ($H = 7.43$, $p = 0.02$) and Chapter 4 (mean ASVs = 15.5; Std Dev = 8.3) and Chapter 3 ($H = 6.01$, $p = 0.02$).

Beta diversity

There was no significant difference in unweighted UniFrac distance between Community and DNA standards (R-statistic = -0.1, $p = 0.8$). However there was a significant difference in weighted UniFrac distance between these groups (R-statistic = 0.88, $p = 0.001$). This suggests that there was no difference in taxonomic composition between Community and DNA standards but that there were differences in relative abundance of these taxonomic components. There were significant differences in beta diversity between community

standards extracted over different experiments (unweighted UniFrac ANOSIM test: R-statistic = 0.19, p = 0.004; weighted UniFrac ANOSIM test: R-statistic = 0.78, p = 0.001) showing differences in taxonomic composition and relative abundance of constituents between community standards from different experiments.

Taxonomic composition and differentially abundant ASVs

The majority of relative abundance in standard samples was composed of the predefined constituents. However, small amounts of contamination from test samples or the environment were detected. On average, contamination formed 0.018% of relative abundance (Std Dev = 0.03).

Gneiss analysis revealed differential ASV abundance between the Community and DNA standards. The ASV table was not filtered and contained a total of 74 ASVs. The overall linear regression model fit was R2 = 0.77 with covariate 'Standard Type' accounting for 2.0% of variance and 'Experiment' accounting for a total of 72.0% of variance (Chapter 3 = 39.0% and Chapter 4 = 33.0%). The log ratio of balance y22 (β = -0.66, $p < 0.001$) was significantly different between Community and DNA standards. Balances y0 (Chapter 3: β = -7.3, $p < 0.001$; Chapter 4: β = 5.3, $p < 0.001$), y1 (Chapter 3: β = 5.1, $p < 0.001$; Chapter 4: β = 5.2, $p < 0.001$), y2 (Chapter 3: β = 8.8, $p < 0.001$; Chapter 4: β = 8.8, $p < 0.001$), y16 (Chapter 3: β = 1.2, p = 0.037; Chapter 4: β = 1.5, p = 0.008) and y22 (Chapter 3: β = 0.5, p = 0.003; Chapter 4: β = 0.4, p = 0.01) were significant predictors for the covariate of 'Experiment'.

The log ratio of balance y22 was lower in Community standards when compared to DNA standards (Figure 2.1). The dendrogram heatmap shows that this was due to an increased abundance of $y22_{\text{numerator}}$ ASVs in DNA standards and an increased abundance of $y22_{\text{denominator}}$ ASVs in Community standards (Figure 2.3). ASVs from $y22_{\text{numerator}}$ were assigned to *Enterococcus* and *Listeria* while those from $y22_{\text{denominator}}$ were assigned to *Escherichia-Shigella*, Enterobacteriaceae and *Pseudomonas*. These differences between Community and DNA standards were further reinforced by examination of relative abundances of the eight taxa forming the standard communities (Table 2.4). The relative

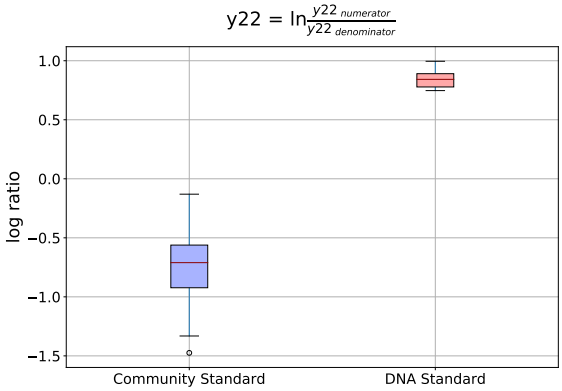
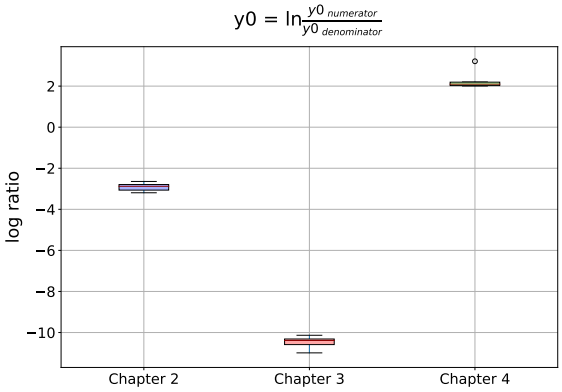


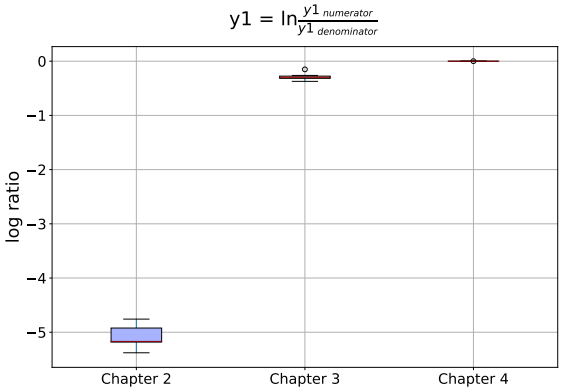
Figure 2.1: Log ratios of balance y22 in Community and DNA standards

abundance of species of Enterobacteriaceae like *Escherichia coli* and *Salmonella enterica* was higher in Community standards when compared using an independent Student's t-test ($t = 7.3$, $p < 0.001$ and $t = 5.5$, $p < 0.001$ respectively). The relative abundance of *Listeria monocytogenes* was significantly lower in Community standards compared to DNA standards ($t = 26.1$, $p < 0.001$).

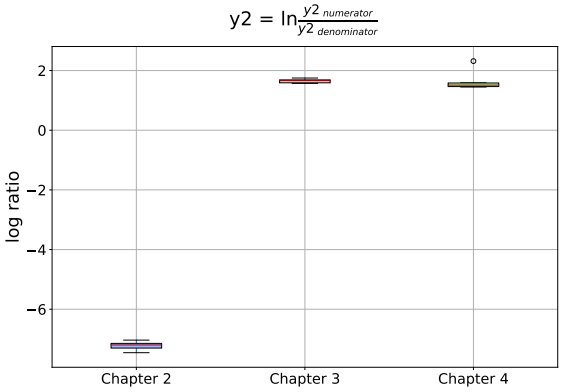
Differences between Community standard composition between experiments was described by balances y0, y1, y2, y16 and y22. The log ratio of balance y0 was lower in Community standards from Chapters 2 and 3 when compared to Chapter 4 (Figure 2.2a). The dendrogram heatmap shows (Figure 2.3) that the abundance of $y0_{\text{denominator}}$ ASVs was higher in standards from Chapters 2 and 3. A further difference in $y0_{\text{denominator}}$ ASV abundance is described by balance y1 in which the log ratio was lower in Community standards from Chapter 2 compared to Community standards from other chapters (Figure 2.2b). The dendrogram heatmap shows that this was due to a low abundance of the $y1_{\text{numerator}}$ ASV in Community standards from Chapter 2. The ASV described by $y1_{\text{numerator}}$ was assigned to *Enterococcus* while the ASV described by $y1_{\text{denominator}}$ was assigned to *Staphylococcus*. The log ratio of balance y2 was lower in Community standards from Chapter 2 (Figure 2.2c) due to a higher abundance of the ASV represented by $y2_{\text{denominator}}$ which was assigned to Enterobacteriaceae. The log ratio of balance y16 was lower in Community standards from Chapter 2 due to a lower abundance of $y16_{\text{numerator}}$ ASVs. These ASVs were assigned to *Escherichia-Shigella*, *Enterococcus*, *Listeria* and *Pseudomonas*. Balance y22 was a



(a) Balance y0

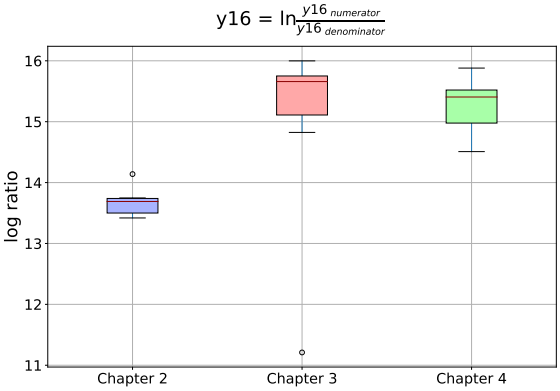


(b) Balance y1

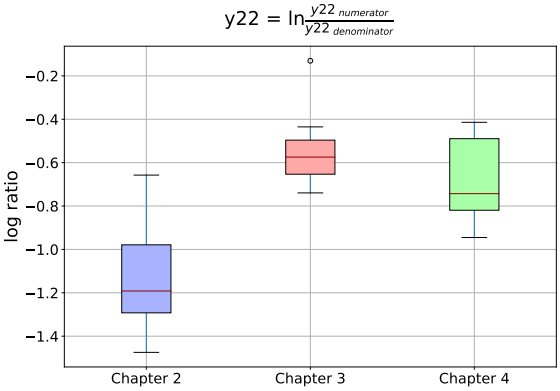


(c) Balance y2

Figure 2.2: Log ratios of the balances significantly different between Community standards from different experiments



(d) Balance y16



(e) Balance y22

Figure 2.2: Log ratios of the balances significantly different between Community standards from different experiments

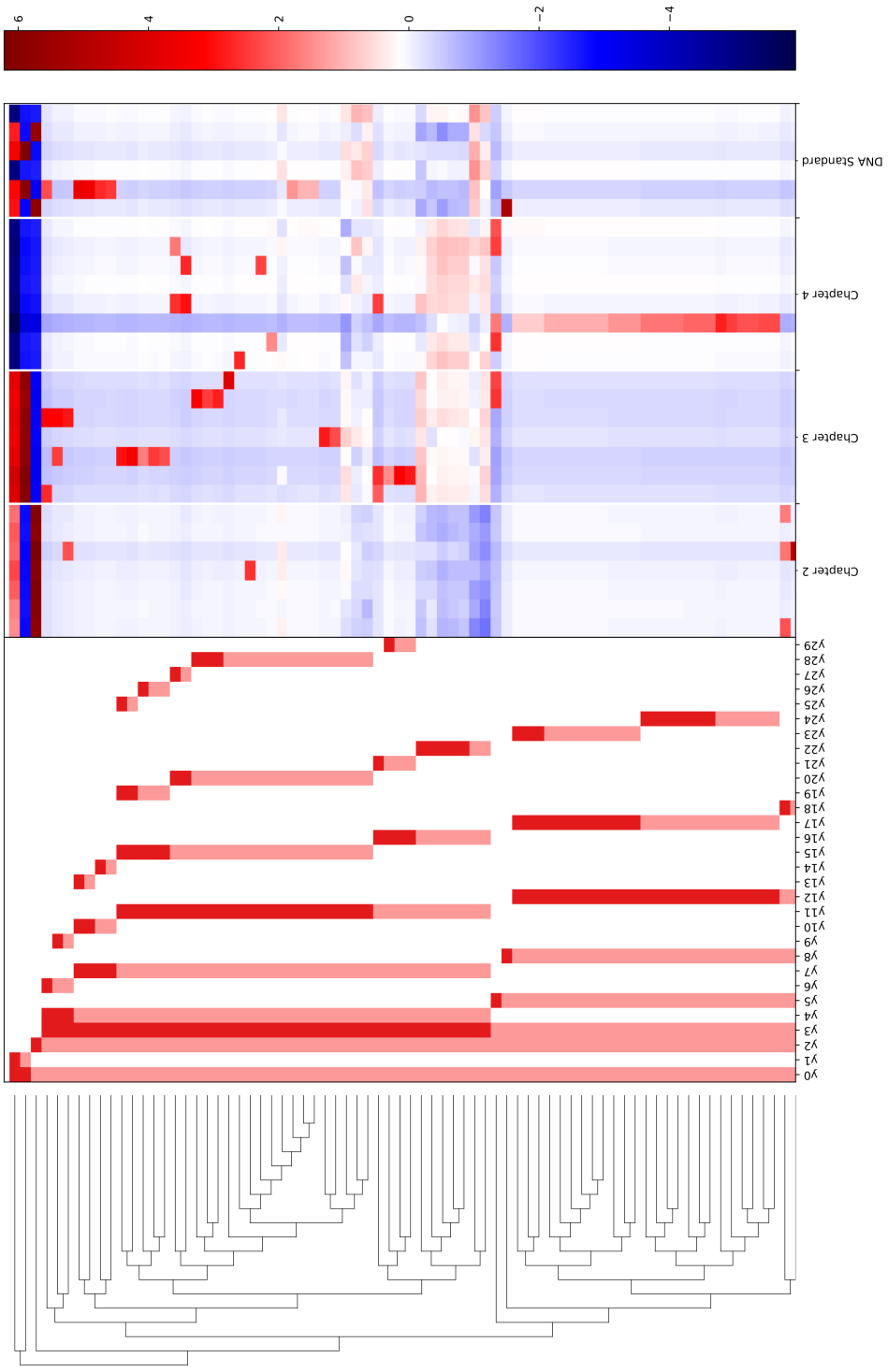


Figure 2.3: ASV log abundance in Community and DNA standards from Chapters 2, 3 and 4

A dendrogram heatmap showing the log abundance of ASVs in Community standards by experiment and DNA standards. Differences in relative abundance between the Community and DNA standards are visibile in balance y22 while differences in Community standards between experiments are visible in balances y0, y1, y2, y16 and y22 which were identified by Gneiss analysis as containing differentially abundant ASVs.

subdivision of $y16_{numerator}$ ASVs and did not add further details to differences in ASV abundance between Community standards from different experiments.

Species	Theoretical Composition (%)		Observed Composition (%)	
	Genomic DNA	16S rRNA gene[1]	DNA Standard	Community Standard
Pseudomonas aeruginosa	12.0	4.6	4.4 (SD = 0.5)	6.8 (SD = 1.3)
Escherichia coli	12.0	10.0	14.1 (SD = 2.4)	27.1 (SD = 5.6)
Salmonella enterica	12.0	11.3	14.1 (SD = 2.1)	23.3 (SD = 2.9)
Lactobacillus fermentum	12.0	18.8	6.6 (SD = 1.0)	6.6 (SD = 3.8)
Enterococcus faecalis	12.0	10.4	10.7 (SD = 1.0)	8.6 (SD = 3.8)
Staphylococcus aureus	12.0	13.3	14.3 (SD = 2.7)	7.2 (SD = 2.0)
Listeria monocytogenes	12.0	15.9	11.6 (SD = 0.8)	3.3 (SD = 0.7)
Bacillus subtilis	12.0	15.7	24.3 (SD = 2.2)	17.2 (SD = 5.0)
Saccharomyces cerevisiae	2.0	-	-	-
Cryptococcus neoformans	2.0	-	-	-

[1] The theoretical composition in terms of 16S rRNA gene abundance was calculated from theoretical genomic DNA composition with the following formula: 16S copy number = total genomic DNA (g) unit conversion constant (bp/g) / genome size (bp) 16S copy number per genome.

Table 2.4: Theoretical and observed composition of Community and DNA Standards

2.3.2 Analysis of pooled compared to individual sample sequencing

Comparing composition of pooled samples to a theoretical average

The abundance of each ASV in a theoretical pooled sample should be the average of the abundance in each constituent sample. In order to create a theoretical pooled sample for each breed, the average abundance for each ASV was calculated and a new column in the ASV table created for this theoretical sample. Taxa plots (Figure 2.4) show that the theoretical composition based on the average composition of the five individual samples was similar to the composition of the pooled sample.

Alpha diversity

A sampling depth of 25,000 was used for diversity metrics. Alpha diversity was measured using an observed ASVs metric. In samples from Hubbard birds, the number of ASVs in the pooled sample (276 ASVs) was similar to the average of individual samples (275 ASVs). For Cobb and Ross birds, pooled samples had a higher number of ASVs (325 and 307 respectively) when compared to the average of individual samples (286 and 207

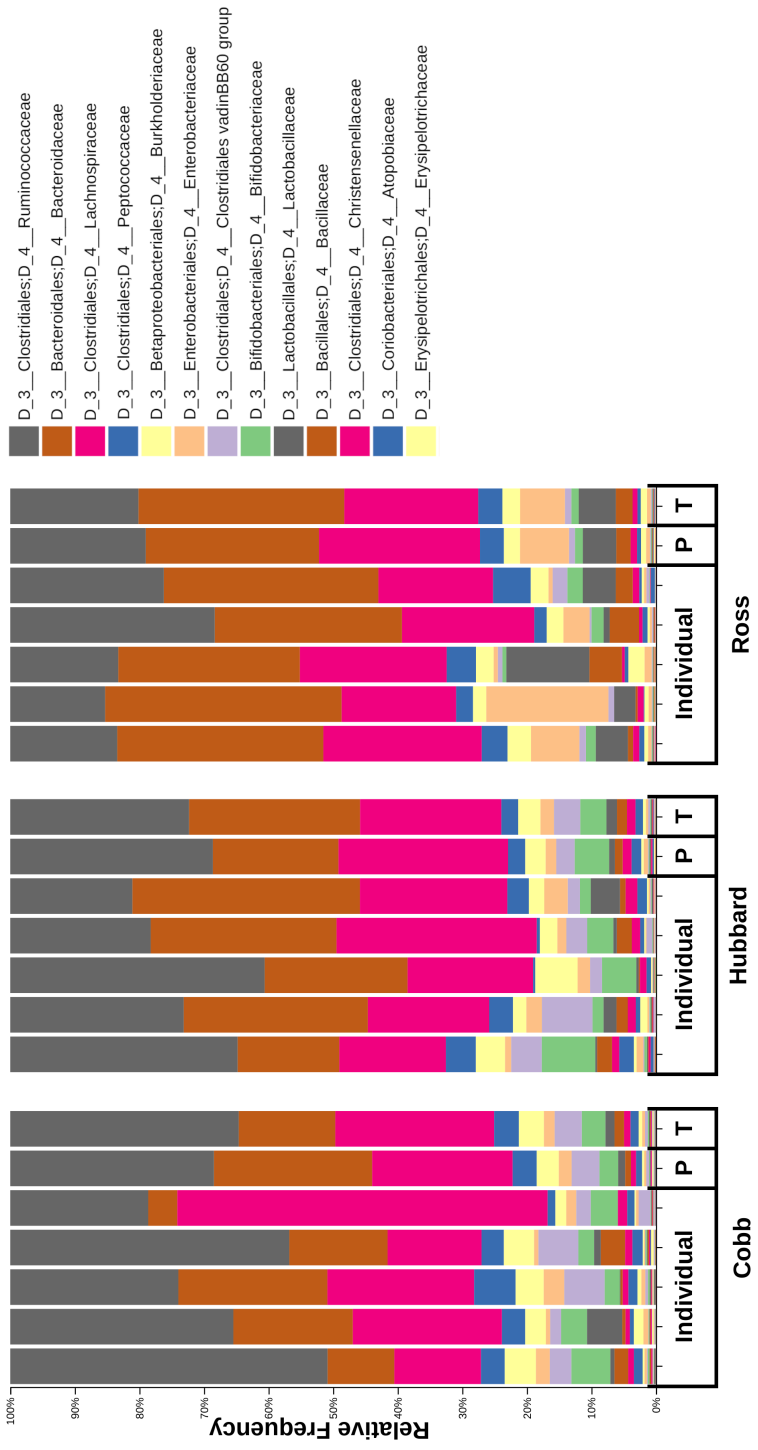


Figure 2.4: Relative abundance of bacterial families in individual, pooled and theoretical average samples of caecal mucus at 42 d.p.h
Pooled (P); Theoretical Average (T)

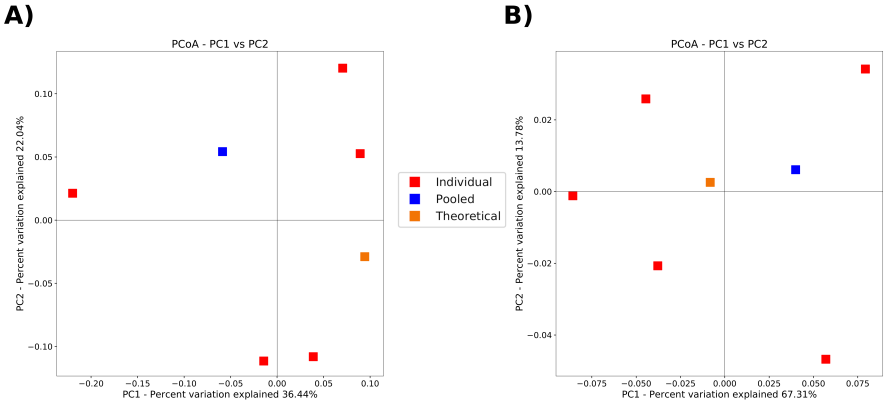
respectively). A higher number of ASVs in pooled samples would be expected as ASVs that are not present in all individual samples will be represented in the pooled sample. However, the alpha diversity of theoretical average samples, which capture all ASVs across the individual samples, shows the diversity which is lost in the process of pooling. In total, 537 ASVs were present in the theoretical sample from Cobb birds with 551 ASVs in the theoretical sample from Hubbard birds and 438 ASVs in the theoretical sample from Ross birds.

The ASV table was reviewed to find features which were present in individual samples but were not present in pooled samples. Most ASVs that were not present in pooled samples were present in only one of the five individual samples. However, a small subset of ASVs which were not present in the pooled sample were present in between three to five of the individual samples.

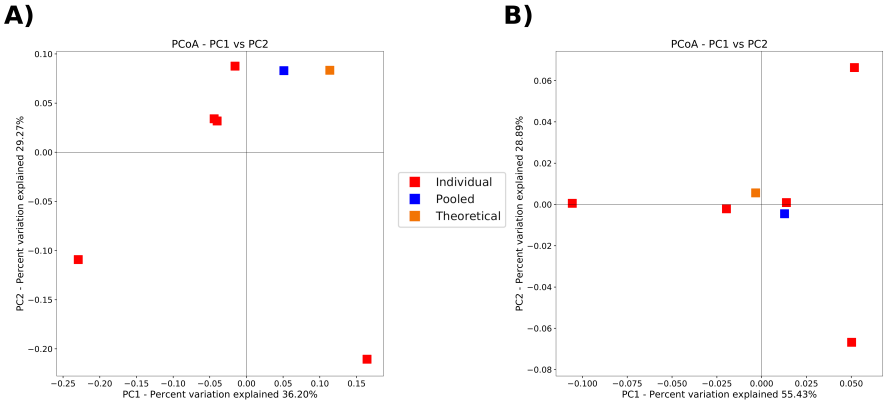
Beta diversity

Beta diversity was calculated separately for each breed using unweighted and weighted UniFrac distances. Individual, theoretical average and pooled samples were included in the analysis. Pooled samples were often closer to certain individual samples than others suggesting that these were over-represented in the pooling.

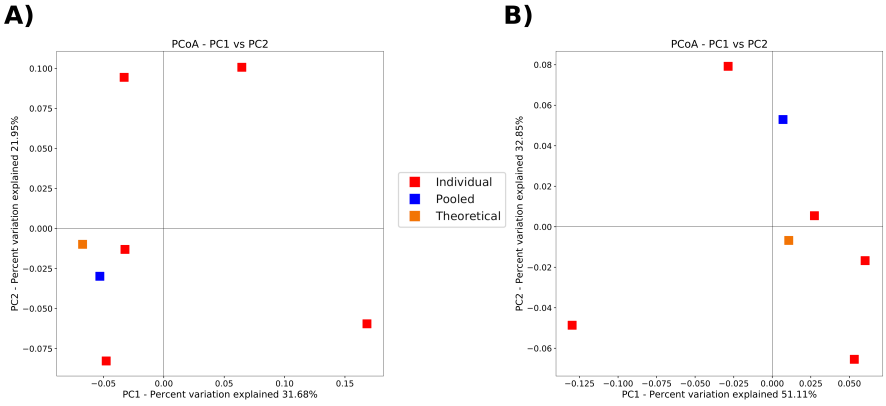
The unweighted and weighted UniFrac distance between each individual sample and its corresponding pooled (IvP) and theoretical average (IvA) samples was investigated further. For unweighted UniFrac distances, there was no significant difference in the average distance between IvP or IvA measured using a paired t-test ($t = 0.04$, $p = 0.96$). Nor was there a significant difference between IvP and IvA weighted UniFrac distances ($t = 1.8$, $p = 0.09$). To examine whether individual samples that were closer to their corresponding pooled sample were also closest to theoretical averages, a Spearman correlation was conducted to compare IvP and IvA distances. This revealed a strong correlation between IvP and IvA unweighted UniFrac distances ($rs(14) = 0.77$, $p < 0.001$) but no significant correlation was detected between IvP and IvA weighted UniFrac distances ($rs(14) = 0.32$, $p = 0.25$).



(a) Hubbard Birds



(b) Ross Birds



(c) Cobb Birds

Figure 2.5: Beta diversity between individual, pooled and theoretical average sample by breed

Beta diversity was measured by unweighted (A) and weighted (B) UniFrac metrics. Across all three breeds pooled samples were closer to some individual samples than others suggesting that pooled samples were more representative of certain constituent samples.

2.3.3 Discussion

Extraction and PCR biases detected using standards

Some degree of contamination was noted in most community standard samples. This is evidenced by higher alpha diversity than would be expected without contamination. Between 11 and 14 ASVs were detected in uncontaminated DNA standards, with the number in Community standards averaging 15 ASVs. The experiment with the highest contamination was that presented in Chapter 3 as evidenced by the significantly higher alpha diversity when compared with other experiments. However, the relative abundance of contaminating features was low, generally contributing less than 0.02% of the relative abundance as a cumulative. It can be concluded that contamination of kits or samples was present but that the effects were limited and should not affect the results presented.

Extraction bias was evidenced by differences in weighted UniFrac distance and relative abundance between Community standards and DNA standards. Across all three experiments, the relative abundance of Enterobacteriaceae was consistently higher in Community standards with a lower abundance of *L. monocytogenes*. Both *E. coli* and *S. enterica* are Gram-negative taxa while *L. monocytogenes* is a Gram-positive taxa. As discussed in Chapter 1.2.2, more vigorous lysis methods are required to successfully lyse all Gram-positive cells due to the presence of a cell wall (Stackebrandt *et al.*, 1999). The DNA extraction protocol used in these experiments included a bead-beating step which could be described as vigorous, however, it's possible that no matter how vigorous the approach to cell lysis, some bias will always be present. The analysis of the Community standards at least shows that the lysis method used was sufficient to lyse Gram-positive cells even if a slight bias was introduced. A longer bead-beating step may have increased lysis efficiency, but would also have increased the levels of sheared DNA which can introduce its own biases during PCR (Stackebrandt *et al.*, 1999; Milling *et al.*, 2004).

Differences in Community standard composition between experiments were also noted with samples from the experiment described in Chapter 2 most different from Chapters 3 and 4. In practical terms this would present an issue if results between experiments were

to be compared directly since the analysis is unable to determine if differences in relative abundance of taxa like *E. coli* were the result of extraction bias. Within experiments, Community standards varied less confirming the validity of the comparisons made between samples from the same experiment.

The relative abundance of *L. fermentum* was far lower in DNA standards compared to their theoretical composition. This difference could be attributed to PCR amplification bias, however, no specific information regarding the differential amplification of *Lactobacillus* sequences could be found in the literature to support this hypothesis. However, it's important to note that the results presented in these experiments may underestimate the relative abundance of *Lactobacillus* spp.

The effect of pooling on sample composition

Alpha diversity showed that some diversity was lost as a consequence of pooling. ASVs which were present in individual samples but not found in pooled samples (excluded ASVs) tended to be present in only one or two individual samples. Excluded ASVs had low relative abundance in samples, defining them as rare taxa. However, despite their low abundance, rare taxa can play essential roles in the ecology and function of the microbiome (Jousset *et al.*, 2017). Since one of the main objectives of 16S rRNA gene sequencing is to capture as much diversity as possible this loss of resolution caused by pooling must be taken into account when analysing results.

PCoA plots showed that pooled samples were more similar to some individual samples than others suggesting that pooling did not represent all constituent samples equally. This may be caused by inadequate homogenisation of the pooled mucus samples. Since mucus is viscous, creating a truly homogeneous pooled sample from which to remove 0.5µl for DNA extraction may be a challenge. A Spearman correlation was used to determine whether individual samples close to their corresponding pooled sample (IvP) were also close to the theoretical average (IvA) sample. A significant correlation in similarity between IvP and IvA distances was found when using an unweighted UniFrac metric suggesting that the pooled samples reflected, to some degree, what would be found with a theoretical

average. However, no such correlation was found when a weighted UniFrac metric was used. This suggests that a weakness of the pooling strategy was that, while major taxonomic composition was accurately reflected, relative abundance was less representative.

This analysis shows that pooling samples can be an effective way of discerning the average composition of a microbial population across several individual samples. However, taxonomic resolution is lost with rare taxa present in few samples often excluded from pooled samples. Additionally, pooling raw sample material may lead to over-representation of certain constituent samples. If pooling is still necessary given financial or methodological considerations, it would be worth exploring whether pooling of extracted DNA would provide more even representation.

2.4 Sequencing Effort

A total of 8,178,042 reads were obtained from 87 experimental samples submitted for sequencing. After filtering, merging of paired reads and chimera removal, a total of 3,691,453 reads remained (45% of the original total) giving a mean of 42,430 reads per sample. The median number of reads per sample was 42,231.

The lowest sequence count was 5,700 obtained from ileal mucus taken from Hubbard broilers at 14 d.p.h. The next lowest sequence count was 16,397. Sequencing depth was set at 16,000 for diversity analyses. While this excluded one sample from the analysis the increased sampling depth was considered less likely to exclude rarer taxa.

2.5 Differences Between the Ileal and Caecal Microbiomes

Alpha diversity

Alpha diversity was measured using FPD index and a SD index (Figure 2.6). The first time point at which comparisons between the ileum and caecum could be made was 3 d.p.h. At this time point, both FPD and SD indices were higher in the caecum although this difference was not significant after correction for multiple tests ($H = 3.86$, $p = 0.08$

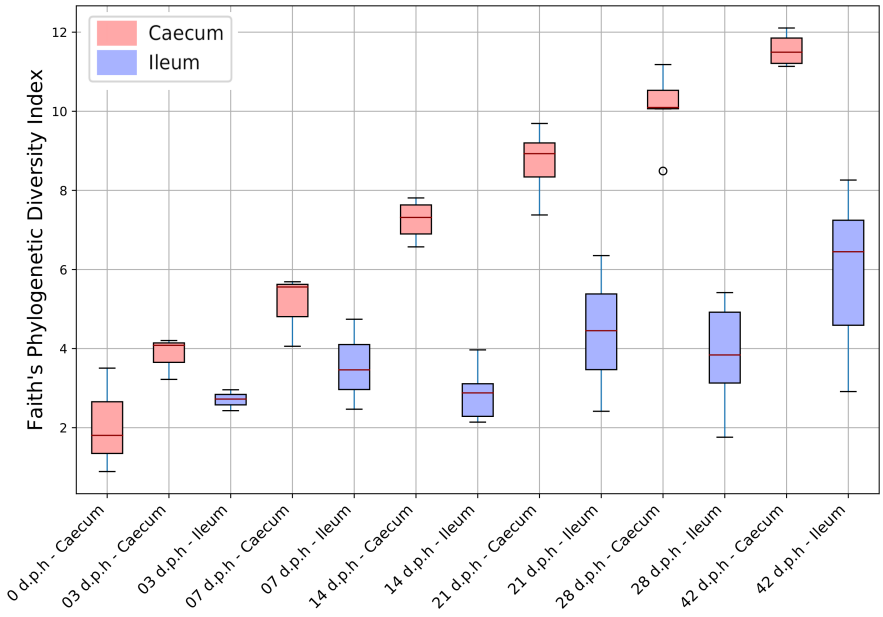
and H = 3.86, p = 0.08 respectively). The same pattern was observed at 7 d.p.h, where a higher FPD and SD index in caecal samples failed to produce a significant result (H = 3.86, p = 0.08 and H = 3.86, p = 0.08 respectively). At 14, 21, 28 and 42 d.p.h, there was a significant difference in alpha diversity between ileal and caecal samples when measured using FPD index (H = 7.50, p = 0.03; H = 8.31, p = 0.02; H = 8.31, p = 0.02 and H = 8.31, p = 0.02 respectively) and a SD index (H = 7.50, p = 0.03; H = 8.31, p = 0.02; H = 8.31, p = 0.02 and H = 8.31, p = 0.02 respectively).

Beta diversity

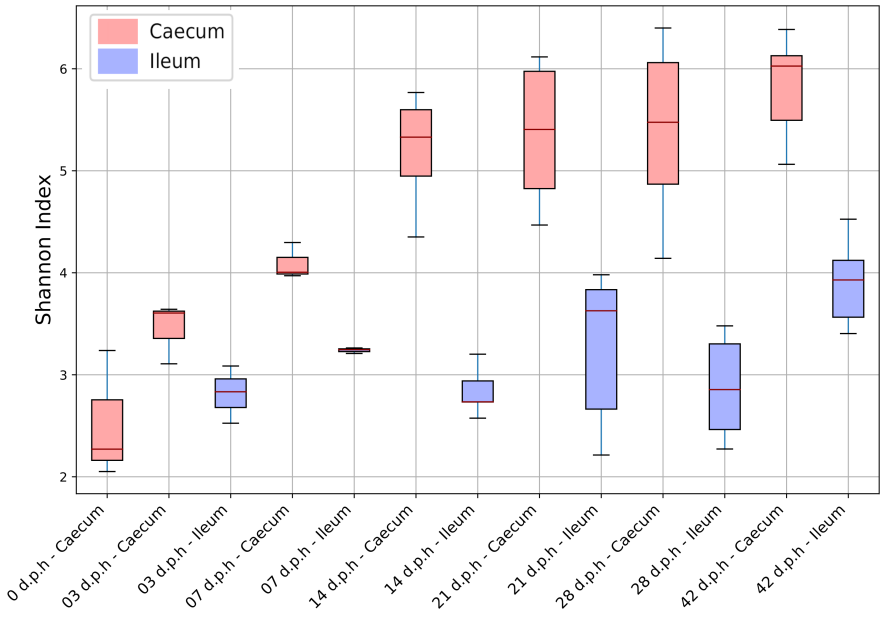
Organ sampled had a significant effect on beta diversity when measured using both unweighted (R = 0.51, p = 0.001) and weighted (R = 0.85, p = 0.001) UniFrac metrics. PCoA plots show clustering of samples by organ although this was time point dependent (Figure 2.7). The clustering of caecal samples at 0 d.p.h was variable with some similarity to both ileal and caecal samples at 3 d.p.h suggesting similarity in microbiome composition between 0 d.p.h caecal samples and 3 d.p.h ileal samples. Samples clustered by organ from 3 d.p.h onwards suggesting that a distinct caecal microbiome was present from between 3 d.p.h. Differences between ileal and caecal samples was more evident using a weighted UniFrac metric compared to an unweighted UniFrac metric.

Differentially abundant ASVs between organs

Gneiss analysis revealed differential ASV abundance between the caecum and the ileum. The ASV table was filtered to exclude ASVs with a frequency of less than 101 reducing the number of ASVs in the analysis from 930 to 467. The overall linear regression model fit was R2 = 0.37 with covariate 'Organ' accounting for 19.6% of variance. The log ratio of balances y0 (β = 21.8, p < 0.001), y2 (β = -13.5, p < 0.001), y6 (β= -10.5, p < 0.001), y13 (β = -5.5, p < 0.001), y28 (β= -5.3, p < 0.001) and y73 (β = 4.2, p < 0.001) were significant predictors for the covariate of 'Organ'. ASVs were classified as having a higher relative abundance in the caecum, ileum or as not differentially abundant (NDA) between the two organs.



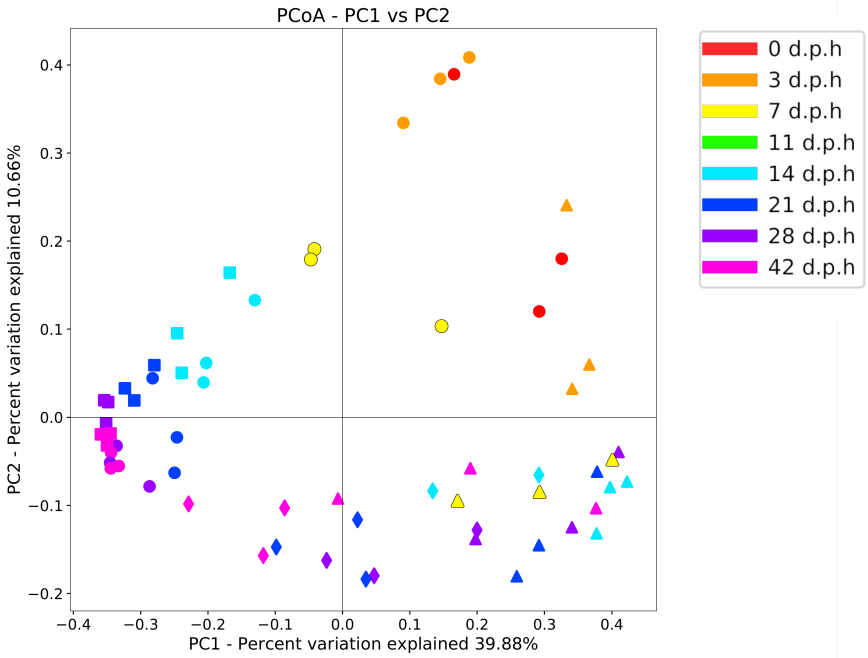
(a) FPD Index



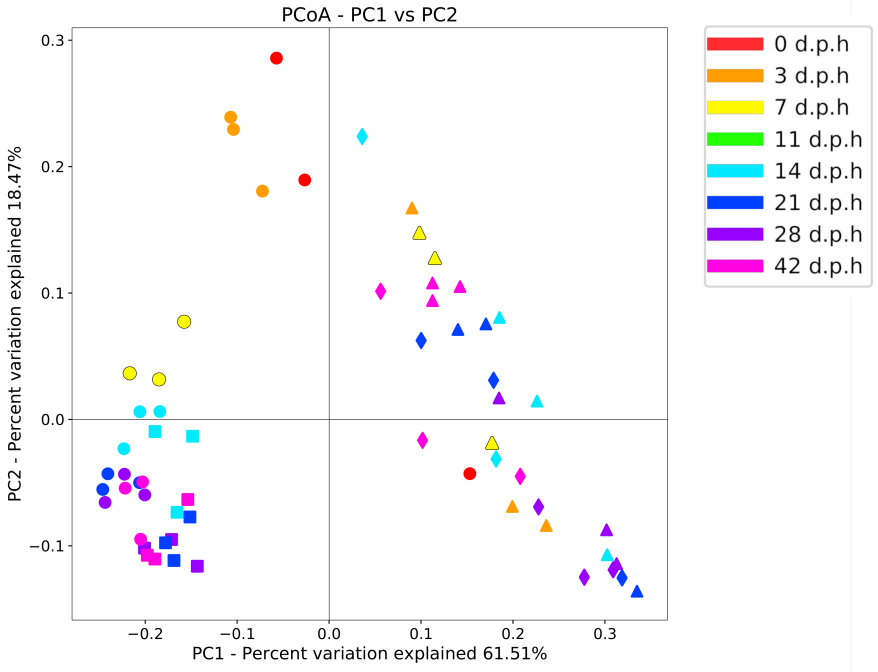
(b) SD Index

Figure 2.6: Alpha diversity of sample groups by organ and time point

Box and whisker plots show the range of alpha diversity measured using FPD and SD metrics. Although the alpha diversity was higher in the caecum at 3 and 7 d.p.h, this difference was not significant. Significant differences in alpha diversity between organs were found from 14 d.p.h.



(a) Unweighted UniFrac



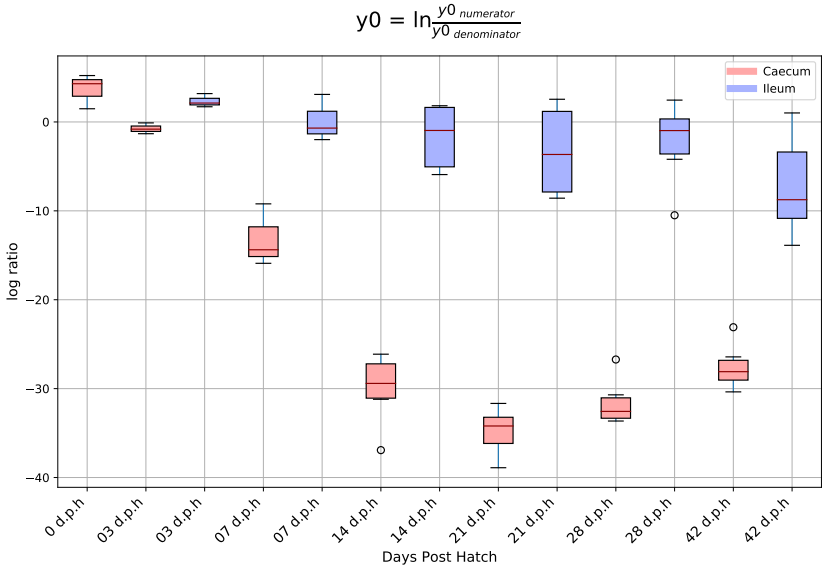
(b) Weighted UniFrac

Figure 2.7: Unweighted and weighted UniFrac beta diversity between caecal and ileal samples

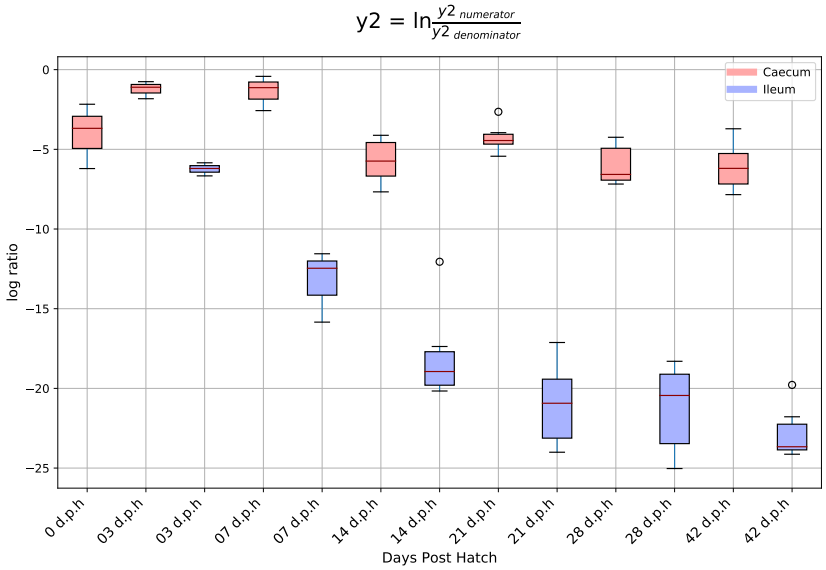
Caecal lumen (●), caecal mucus (■), ileal lumen (▲) and ileal mucus (♦)

The log ratio of balance y0 was lower in caecal samples compared to ileal samples with the effect most accentuated at later time points (Figure 2.8a). The dendrogram heatmap (Figure 2.9) shows that this was due to an increased relative abundance of $y0_{denominator}$ ASVs in caecal samples. Further balances are required to determine which $y0_{numerator}$ ASVs were differentially abundant. Balance y2 is a subdivision of $y0_{numerator}$ ASVs. The log ratio of balance y2 was lower in ileal samples (Figure 2.8b). The dendrogram heatmap shows that an increased relative abundance of $y2_{denominator}$ ASVs explains this pattern with further balances required to determine whether $y2_{numerator}$ ASVs were more abundant in either the caecum or ileum. Balance y6 is a subdivison of $y2_{numerator}$ ASVs. The log ratio of balance y6 was lower in caecal samples (Figure 2.8c) with the dendrogram heatmap showing an increased relative abundance of $y6_{numerator}$ ASVs in caecal samples. Further balances are required to determine differential abundance among $y6_{denominator}$ ASVs. Balance y13 is a subdivision of $y6_{denominator}$ ASVs. The log ratio of balance y13 was lower in ileal samples at most time points (Figure 2.8d). The dendrogram heatmap shows a higher relative abundance of $y13_{denominator}$ ASVs in ileal samples. Balance y28 is a subdivision of $y13_{numerator}$ ASVs. The log ratio of balance y28 was lower in ileal samples (Figure 2.8e). The dendrogram heatmap shows a higher relative abundance of $y28_{denominator}$ ASVs in ileal samples. Balance y48 is a subdivision of $y28_{numerator}$ ASVs, however there are no significant differences between ileal and caecal samples at this balance. The dendrogram heatmap shows that $y48_{denominator}$ ASVs were associated with an early microbiome but were not differentially abundant between ileal and caecal samples. However, balance y73 is a subdivision of $y48_{numerator}$ ASVs. The log ratio of balance y73 was lower in caecal samples (Figure 2.8f). The dendrogram heatmap shows that while $y73_{denominator}$ ASVs were rare, the relative abundance was higher in caecal samples while $y73_{numerator}$ ASVs were associated with the early microbiome and were not differentially abundant between organs.

The taxonomy of ASVs identified as differentially abundant in the caecum and ileum is displayed in Table 2.5. Almost all ASVs assigned to Ruminococcaceae, Lachnospiraceae and Clostridiales vadin BB60 group were more abundant in the caecum. All ASVs assigned to Bacillaceae, Eggerthellaceae, Peptococcaceae, Burkholderiaceae, Bacteroidaceae, Chris-

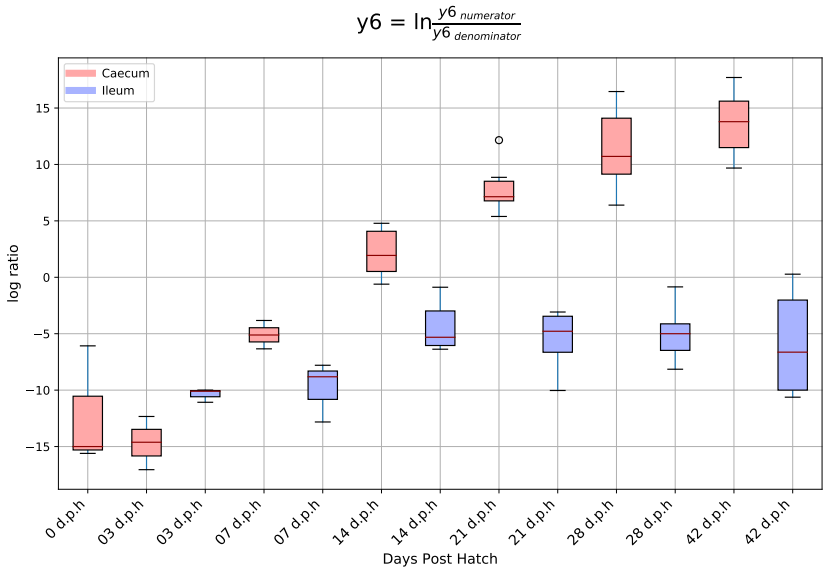


(a) Balance y0

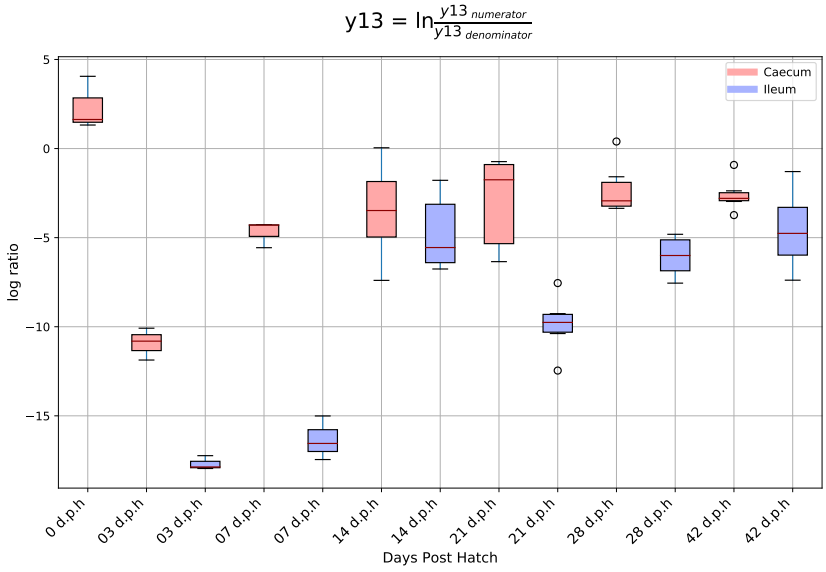


(b) Balance y2

Figure 2.8: Log ratios of balances significantly different between the ileum and caecum

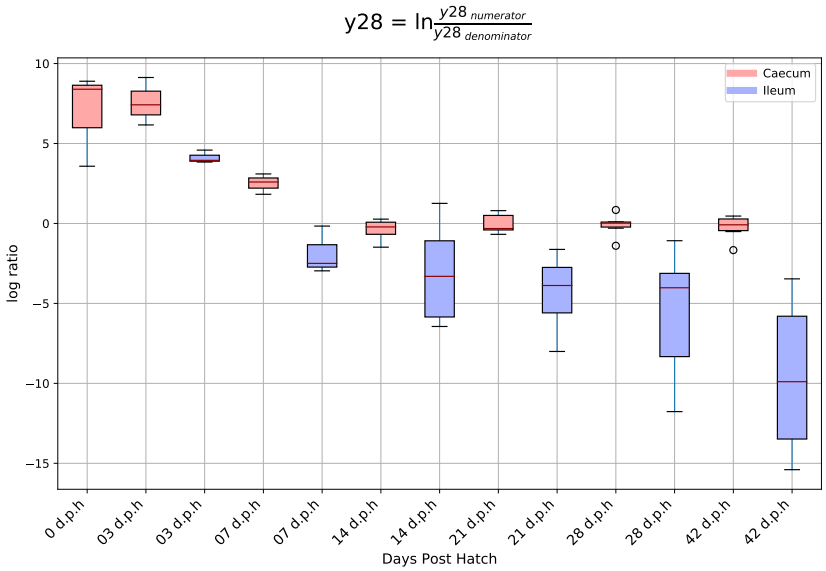


(c) Balance y6

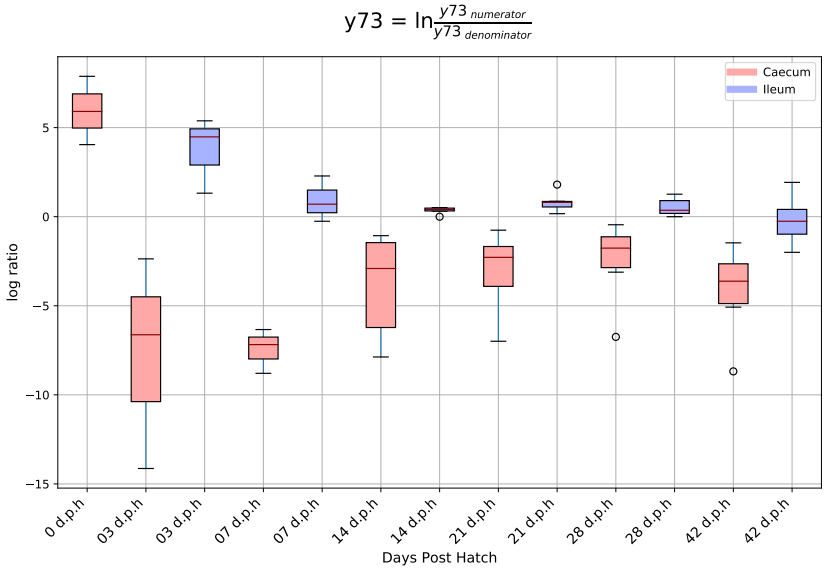


(d) Balance y13

Figure 2.8: Log ratios of balances significantly different between the ileum and caecum



(e) Balance y28



(f) Balance y73

Figure 2.8: Log ratios of balances significantly different between the ileum and caecum

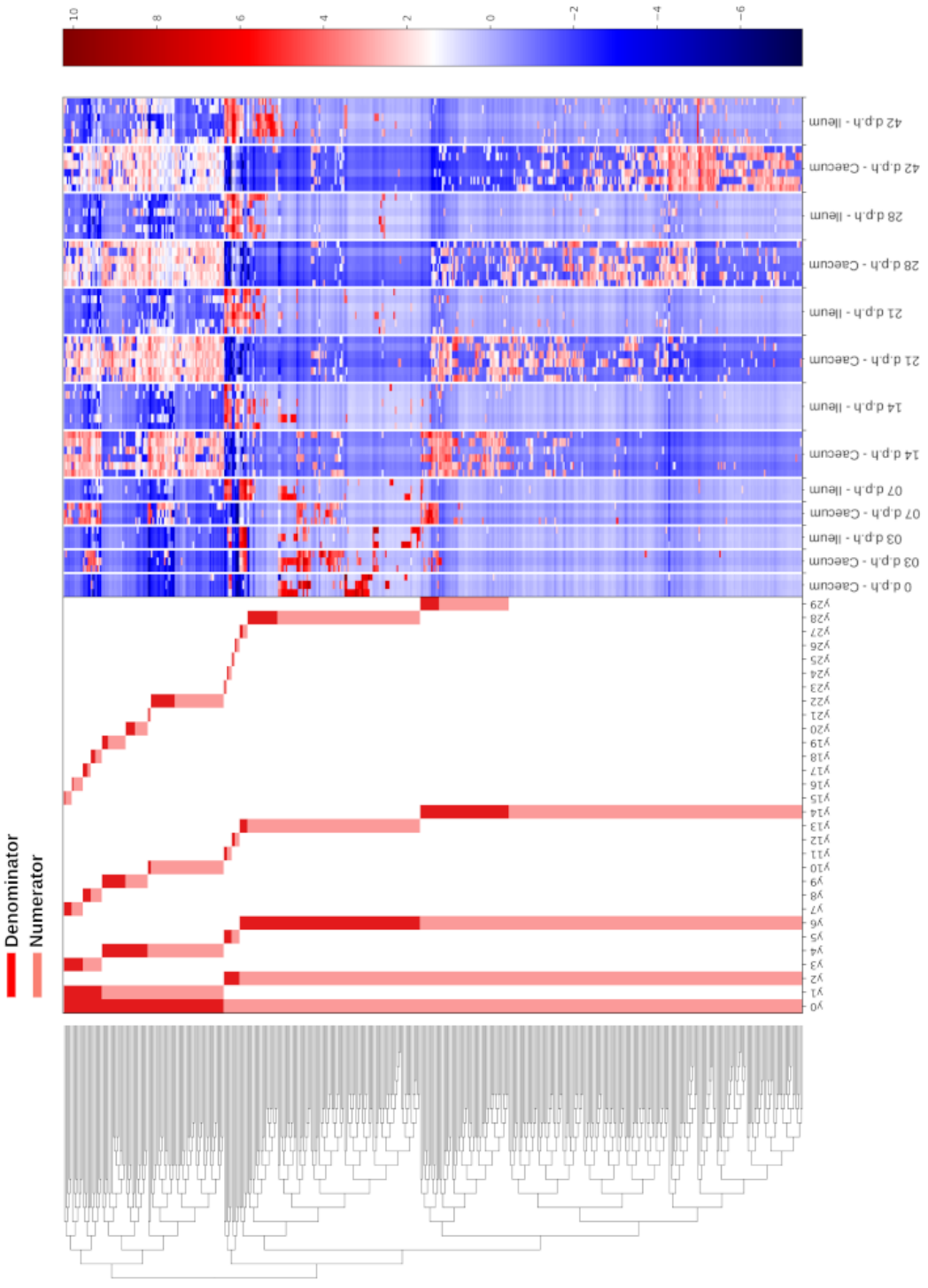


Figure 2.9: ASV log abundance in the ileal and caecal microbiomes

A dendrogram heatmap showing the log abundance of ASVs in samples grouped by organ and age. Differences in relative abundance between the ileum and caecum are visible in balances y0, y2, y6, y13 and y28 identified by Gneiss analysis as containing differentially abundant ASVs.

taxonomy assignment.

		Number of ASVs		
Taxonomy	**Total**	**Caecum**	**Ileum**	**NDA** [a]
Ruminococcaceae	165	162	0	3
Lachnospiraceae	122	115	1	6
Clostridiales vadin BB60 group	29	28	0	1
Clostridiaceae 1	23	1	3	19
Enterococcaceae	23	1	6	16
Enterobacteriaceae	19	2	2	15
Peptostreptococcaceae	12	0	8	4
Lactobacillaceae	10	2	7	1
Christensenellaceae	10	10	0	0
Erysipelotrichaceae	6	4	2	0
Bacillaceae	5	5	0	0
Eggerthellaceae	5	5	0	0
Peptococcaceae	4	4	0	0
Burkholderiaceae	4	4	0	0
Bacteroidaceae	3	3	0	0
Coriobacteriaceae	3	3	0	0
uncultured rumen bacterium	2	2	0	0
Atopobiaceae	2	2	0	0
Eubacteriaceae	2	2	0	0
Family XIII	2	2	0	0
Corynebacteriaceae	2	0	1	1
Staphylococcaceae	2	0	2	0
Aerococcaceae	2	0	1	1
Clostridiales	2	2	0	0
Streptococcaceae	1	0	0	1
Bifidobacteriaceae	1	1	0	0
Defluviitaleaceae	1	1	0	0
gut metagenome	1	1	0	0
Mollicutes RF39	1	1	0	0
Firmicutes bacterium CAG:822	1	1	0	0
Dermabacteraceae	1	0	0	1
Planococcaceae	1	0	1	0

[a] ASVs defined as NDA were not differentially abundant between caecal and ileal samples. Individual taxonomies of significant Gneiss balances are provided in Table A.2.

Table 2.5: Taxonomy of differentially abundant ASVs between the caecum and ileum

Differences in composition between the caecal and ileal microbiomes can be seen in the taxa plots (Figure 2.10). The caecal microbiome was mainly composed of Bacteroidaceae,

Lachnospiraceae and Ruminococcaceae. Less common taxa associated with the caecal microbiome included Burkholderiaceae, Clostridiales vadin BB60 group, Atopobiaceae, Bacillaceae, Peptococcaceae, Christensenellaceae, Coriobacteriaceae, Eubacteriaceae, Eggerthellaceae and Mollicutes RF39. The ileal microbiome was mainly composed of Lactobacillaceae, Enterococcaceae, Erysipelotrichaceae, Peptostreptococcaceae and Clostridiaceae 1. Many ASVs in the ileum at later time points which were assigned to Clostridiaceae 1 were identified as *Candidatus* Arthromitusat the genus level. Other less common taxa associated with the ileal microbiome included Staphylococcaceae and Aerococcaceae.

2.5.1 Discussion

The comparison of the caecal and ileal microbiomes produced similar results to that of previous studies. The ileal and caecal microbiota were most similar at 3 d.p.h although significant differences in composition were noted between organ microbiotas at this early time point. The similarity of the microbiomes between organs cannot be directly compared at 0 d.p.h, however, samples from the ileum at 3 d.p.h clustered with samples from the caecum at 0 d.p.h on unweighted UniFrac beta diversity plots suggesting a similar microbiome composition. By 7 d.p.h the caecum and ileum were inhabited by distinct bacterial communities. This is in accordance with previous observations that the caecal and ileal microbiomes begin to diverge from approximately 3 d.p.h (Van Der Wielen *et al.*, 2002; Lu *et al.*, 2003). The ASVs that inhabited the caecum and ileum at 0 and 3 d.p.h were identified by Gneiss analysis as not differentially abundant between organs. At the genus level, these ASVs were assigned to *Clostridium sensu stricto* 1, *Escherichia-Shigella* and *Enterococcus*. This is similar to the pattern of colonisation described by other authors and raises the same concerns about early colonisation of chicks by possible pathogens (Johnson *et al.*, 2018; Jurburg *et al.*, 2019).

The caecal microbiome was significantly more diverse than the ileal microbiome both in terms of the number of ASVs and their phylogenetic diversity. As expected, the caecum was populated by an assortment of Firmicutes with Clostridiales such as Lachnospiraceae and Ruminococcaceae being the most common taxonomic assignment in this phylum.

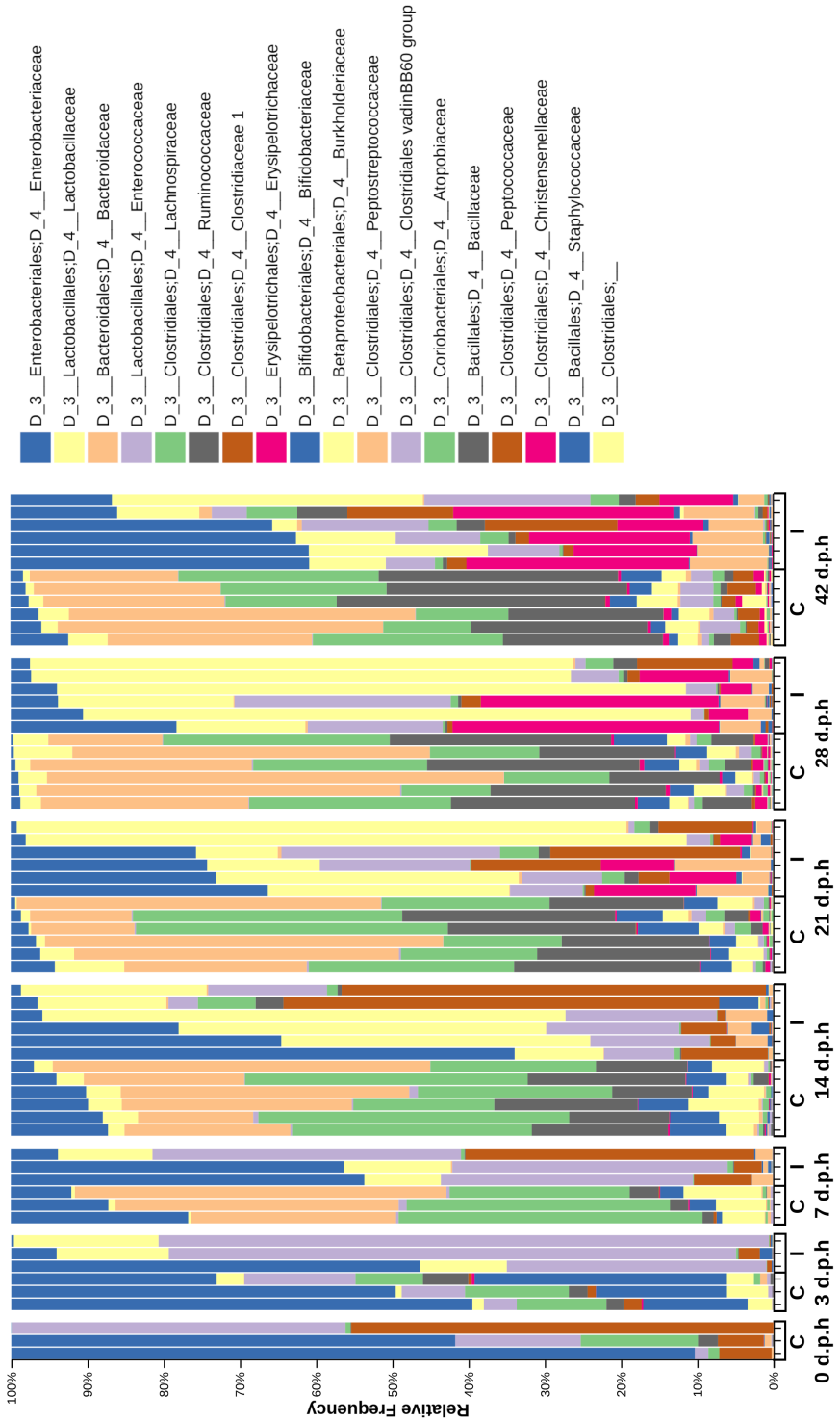


Figure 2.10: Relative abundance of bacterial families in the caecum and ileum between 0 and 42 d.p.h

Caecum (C); Ileum (I)

Other inhabitants of the caecal microbiome included Bacteroidaceae, Bifidobacteriaceae, Coriobacteriaceae, Eggerthellaceae and Burkholderiaceae which have all been previously reported (Johnson *et al.*, 2018; Borda-Molina *et al.*, 2016; Jurburg *et al.*, 2019; Pandit *et al.*, 2018). Similarly, the taxa which were differentially abundant in the ileum such as Lactobacillaceae, *Candidatus* Arthromitus, Peptostreptococcaceae, Erysipelotrichaceae and Staphylococcaceae are often reported as members of the ileal microbiota (Johnson *et al.*, 2018; Latorre *et al.*, 2018; Kollarcikova *et al.*, 2019). However, when considered at the genus level, some unreported differences between organs were observed. For example, within Peptostreptococcaceae only the genus *Romboutsia* was consistently more abundant in the ileum whereas *Clostridioides* was split between higher abundance in the ileum and no differential abundance between organs. Equally, Erysipelotrichaceae was divided into *Turicibacter*, which was more abundant in the ileum, and *Faecalicoccus* and *Erysipelatoclostridium* which were more abundant in the caecum. Two ASVs assigned to Lactobacillaceae were found to be differentially abundant in the caecum, including the only ASV assigned to *Pediococcus*. However, a review of the relative abundance showed that this ASV formed less than 0.5% of total relative abundance at 7 d.p.h before disappearing from the microbiome. As such, the importance of this ASV to the microbiome appears to be negligible. However, the possibility that strains of Lactobacillaceae, a common probiotic taxa, may be more adapted for colonisation of the caecum than the ileum should be considered.

2.6 The Ileal Microbiome

The development of the ileal microbiome between 3 and 42 d.p.h was observed in Hubbard, Ross and Cobb chickens. Lumen samples were taken from 3 d.p.h and mucus samples from 14 d.p.h. As before, sampling depth was set at 16,000 for diversity analyses. While this excluded the mucus sample taken from Hubbard birds at 14 d.p.h the increased sampling depth was considered less likely to exclude rarer taxa.

2.6.1 Succession in the ileal microbiome

Alpha diversity

Overall, age had a significant effect on alpha diversity when measured using FPD (H = 11.4, p = 0.044) and SD index (H = 12.6, p = 0.027). Although there was an increase in alpha diversity at 42 d.p.h (Figure 2.11) there were no significant differences found using pairwise comparisons of alpha diversity between time points.

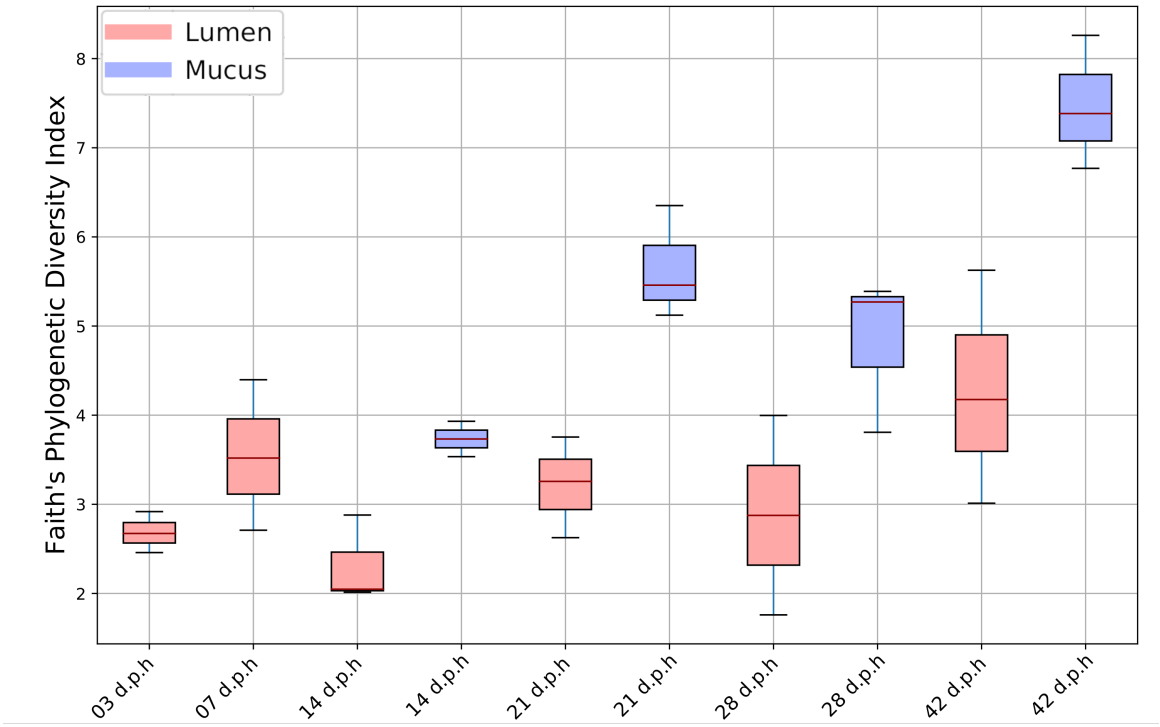
Beta diversity

Age had a significant effect on beta diversity when measured with an unweighted and weighted UniFrac metric (R = 0.30, p = 0.002 and R = 0.25, p = 0.008 respectively). When observed using an unweighted UniFrac metric, samples from 3 d.p.h formed a distinct cluster away from other samples. From 7 d.p.h, there was no apparent pattern of clustering by time point with samples from all time points mixing together (Figure 2.12a). When observed using a weighted UniFrac metric, there was no clustering pattern by time point (Figure 2.12b).

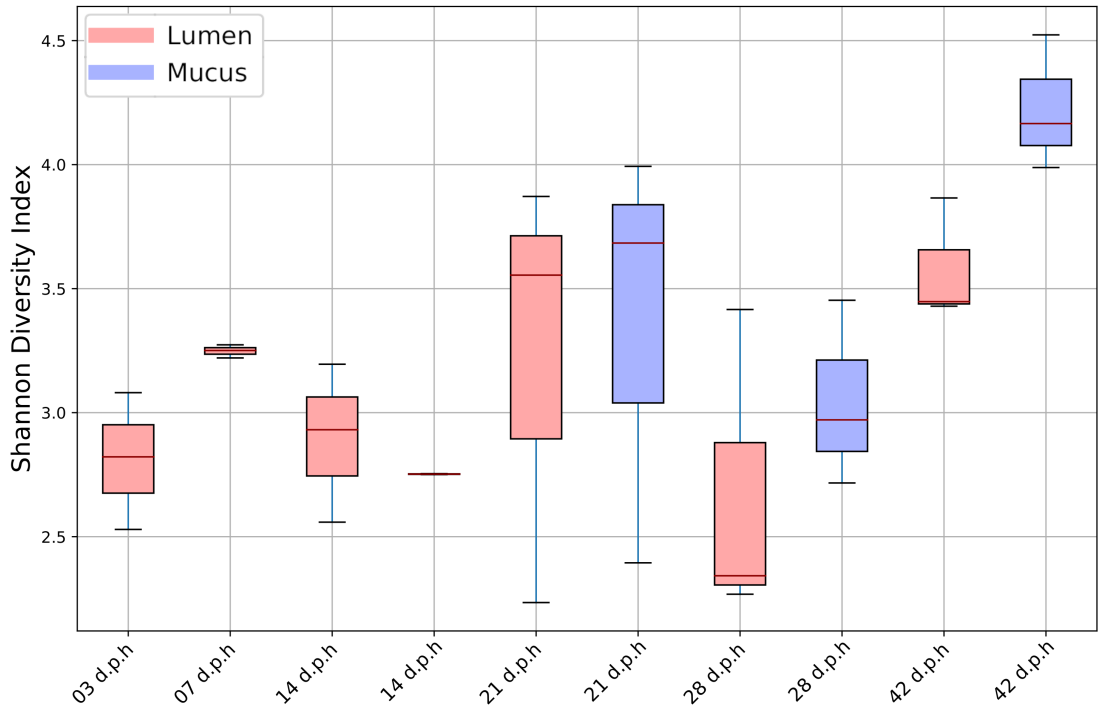
Taxonomic composition

The composition of the microbiome at different time points was observed in a taxa plot (Figure 2.13). At 3 d.p.h, Enterococcaceae was the most abundant taxa in Cobb birds and Hubbard birds (74.4% and 80.1% respectively) while Enterobacteriaceae was the most abundant in Ross birds (53.7%). Lactobacillaceae was present in all three breeds (Cobb birds = 14.7%, Hubbard birds = 19.0%, Ross birds = 11.3%). Small proportions of Bifidobacteriaceae (Cobb birds = 1.6%, Hubbard birds = 0.22%, Ross birds = 0.18%) and Clostridiaceae 1 (Cobb birds = 2.8%, Hubbard birds = 0.13%, Ross birds = 0.6%), further classified as *Clostridium sensu stricto* 1 at the genus level, were found in all three breeds.

At 7 d.p.h, the relative abundance of Lactobacillaceae (Cobb birds = 10.0%, Hubbard birds = 14.0%, Ross birds = 12.4%) and Enterococcaceae (Cobb birds = 33.1%, Hubbard birds = 36.1%, Ross birds = 40.5%) were similar between breeds. There was a higher



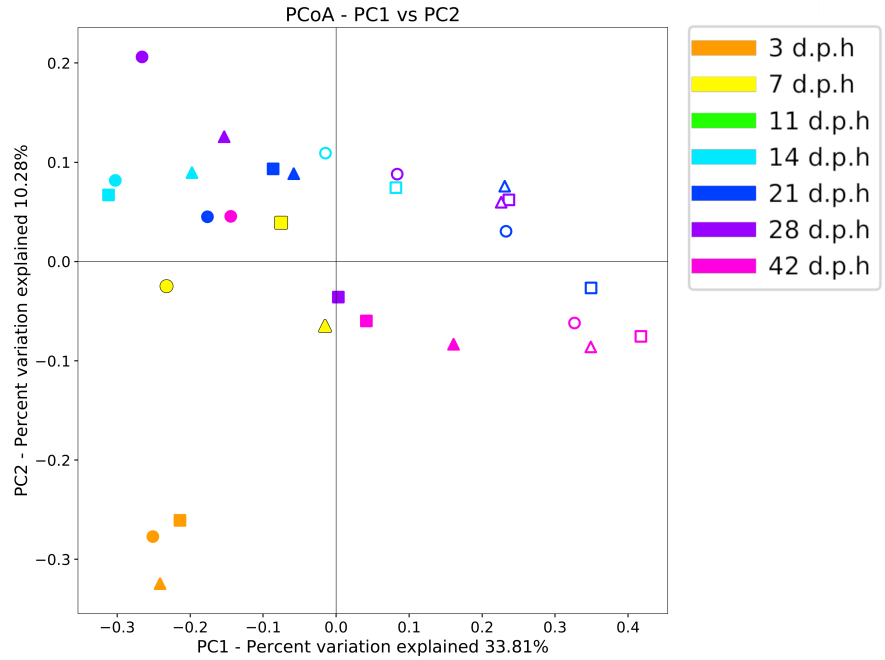
(a) FPD Index



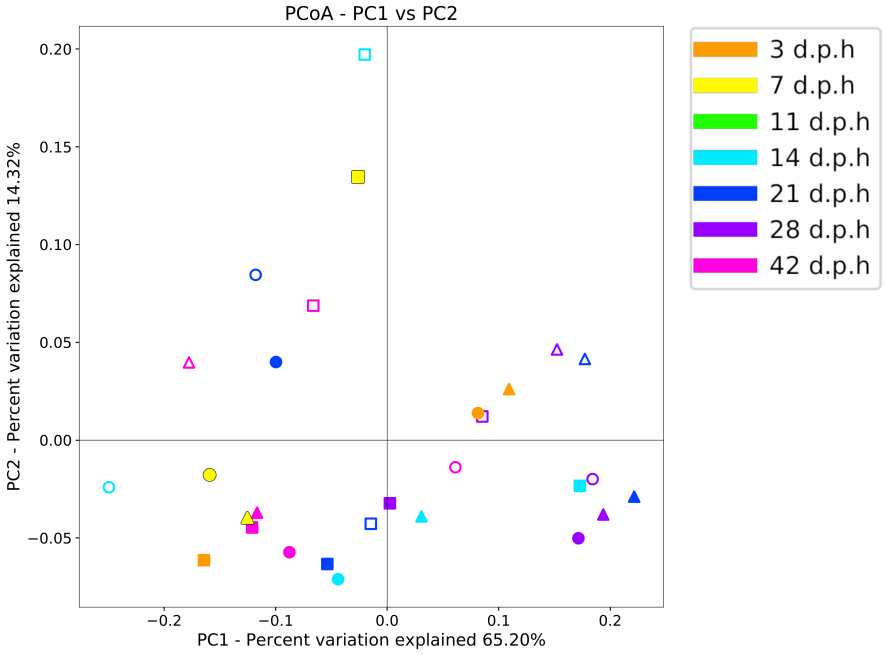
(b) SD Index

Figure 2.11: Alpha diversity in the ileum from 3 to 42 d.p.h

Although there was a significant overall effect of age on alpha diversity there were no significant differences in alpha diversity between time points in the ileum.



(a) Unweighted UniFrac



(b) Weighted UniFrac

Figure 2.12: Beta diversity in the ileum from 3 to 42 d.p.h

Samples from Hubbard (▲), Ross (■) and Cobb (●) chickens are shown with lumen (filled) and mucus (no fill) samples denoted by colour fill.

relative abundance of Enterobacteriaceae in Cobb birds and Hubbard birds compared to Ross birds (Cobb birds = 46.4%, Hubbard birds = 43.7%, Ross birds = 6.2%) while Clostridiaceae 1, further classified as *Candidatus* Arthromitusat the genus level, was more abundant in Ross birds (Cobb birds = 7.6%, Hubbard birds = 3.6%, Ross birds = 37.9%). Peptostreptococcaceae was also present in all three breeds (Cobb birds = 2.7%, Hubbard birds = 0.57%, Ross birds = 2.2%).

At 14 d.p.h, Lactobacillaceae was the most abundant taxa in the lumen (Cobb birds = 40.5%, Hubbard birds = 48.2%, Ross birds = 68.6%). In ileal mucus, the most abundant taxa was Candidatus *Athromitus* in Ross birds (55.7%) and Hubbard birds (57.2%) with Enterobacteriaceae the most abundant in ileal mucus from Cobb birds (66.1%). Peptostreptococcaceae (Cobb birds = 4.2%, Hubbard birds = 3.1%, Ross birds = 5.3%) and Staphylococcaceae (Cobb birds = 0.59%, Hubbard birds = 2.3%, Ross birds = 0.77%) were present in the lumen.

At 21 d.p.h, samples from Ross and Cobb birds had a similar composition with Lactobacillaceae (Cobb birds = 14.8%, Ross birds = 31.7%), Enterococcaceae (Cobb birds = 19.7%, Ross birds = 9.5%), Enterobacteriaceae (Cobb birds = 25.7%, Ross birds = 33.7%), and Erysipelotrichaceae (Cobb birds = 9.5%, Ross birds = 13.3%) the most abundant taxa in the lumen. Lactobacillaceae was the most abundant taxa in lumen samples from Hubbard birds (86.7%) with a lower relative abundance of Enterococcaceae (3.1%), Enterobacteriaceae (1.9%) and Erysipelotrichaceae (4.2%). The composition of the mucus was similar to that of the lumen but a higher relative abundance of *Candidatus* Arthromituswas present (Cobb birds = 25.0%, Hubbard birds = 12.4%, Ross birds = 4.1%). Lachnospiraceae (Cobb birds = 5.1%, Hubbard birds = 2.1%, Ross birds = 3.0%) and Ruminococcaceae (Cobb birds = 1.5%, Hubbard birds = 1.1%, Ross birds = 1.8%) were present in the mucus at higher relative abundances than previously observed.

At 28 d.p.h, Lactobacillaceae was the most abundant taxa in lumen samples from Cobb and Hubbard birds (79.7% and 82.5% respectively) resulting in lower relative abundances of Enterococcaceae (1.7% and 3.9% respectively), Enterobacteriaceae (9.5% and 6.1% respectively) and Erysipelotrichaceae (5.1% and 4.1% respectively). Lumen samples from

Ross birds had a lower relative abundance of Lactobacillaceae (16.9%) and a higher relative abundance of Enterococcaceae (17.7%), Enterobacteriaceae (21.7%) and Erysipelotrichaceae (35.0%). Peptostreptococcaceae was present in all lumen samples (Cobb birds = 3.1%, Hubbard birds = 2.1%, Ross birds = 5.3%). The composition of mucus samples was similar to that of lumen samples.

At 42 d.p.h, there was a similar taxonomic composition between breeds with Lactobacillaceae (Cobb birds = 23.5%, Hubbard birds = 13.1%, Ross birds = 10.0%), Enterococcaceae (Cobb birds = 9.4%, Hubbard birds = 11.1%, Ross birds = 6.5%), Enterobacteriaceae (Cobb birds = 39.1%, Hubbard birds = 37.5%, Ross birds = 39.2%), Erysipelotrichaceae (Cobb birds = 16.1%, Hubbard birds = 21.1%, Ross birds = 29.2%) and Peptostreptococcaceae (Cobb birds = 9.4%, Hubbard birds = 9.2%, Ross birds = 10.2%) forming the major taxa in the ileal lumen. The composition of the mucus microbiome was similar to that of the lumen but higher relative abundances of *Candidatus* Arthromitus(Cobb birds = 3.1%, Hubbard birds = 17.4%, Ross birds = 13.9%), Lachnospiraceae (Cobb birds = 3.7%, Hubbard birds = 3.7%, Ross birds = 6.6%) and Ruminococcaceae (Cobb birds = 2.2%, Hubbard birds = 3.7%, Ross birds = 6.6%) were observed.

Differentially abundant ASVs

Gneiss analysis revealed differential ASV abundance from 3 to 42 d.p.h in the ileum. The ASV table was filtered to exclude ASVs with a frequency of less than 29 reducing the number of ASVs in the analysis from 391 to 201. The overall linear regression model fit was R2 = 0.59 with covariates '07 d.p.h' , '14 d.p.h', '21 d.p.h', '28 d.p.h' and '42 d.p.h' accounting for 6.3%, 10.7%, 10.8%, 12.9% and 16.1% of variance respectively. The log ratio of balances y0, y1, y2, y6, y10, y11 and y18 were significant predictors for one or more time points.

The log ratio of balance y0 was significantly lower at 14 (β = -10.9, $p < 0.001$), 21 (β = -16.3, $p < 0.001$), 28 (β = -19.0, $p < 0.001$) and 42 (β = -17.3, $p < 0.001$) d.p.h demonstrating that $y0_{denominator}$ ASVs were associated with a later microbiome (Figure 2.14a). Balance y1, a subdivision of balance $y0_{denominator}$ ASVs, was significantly higher

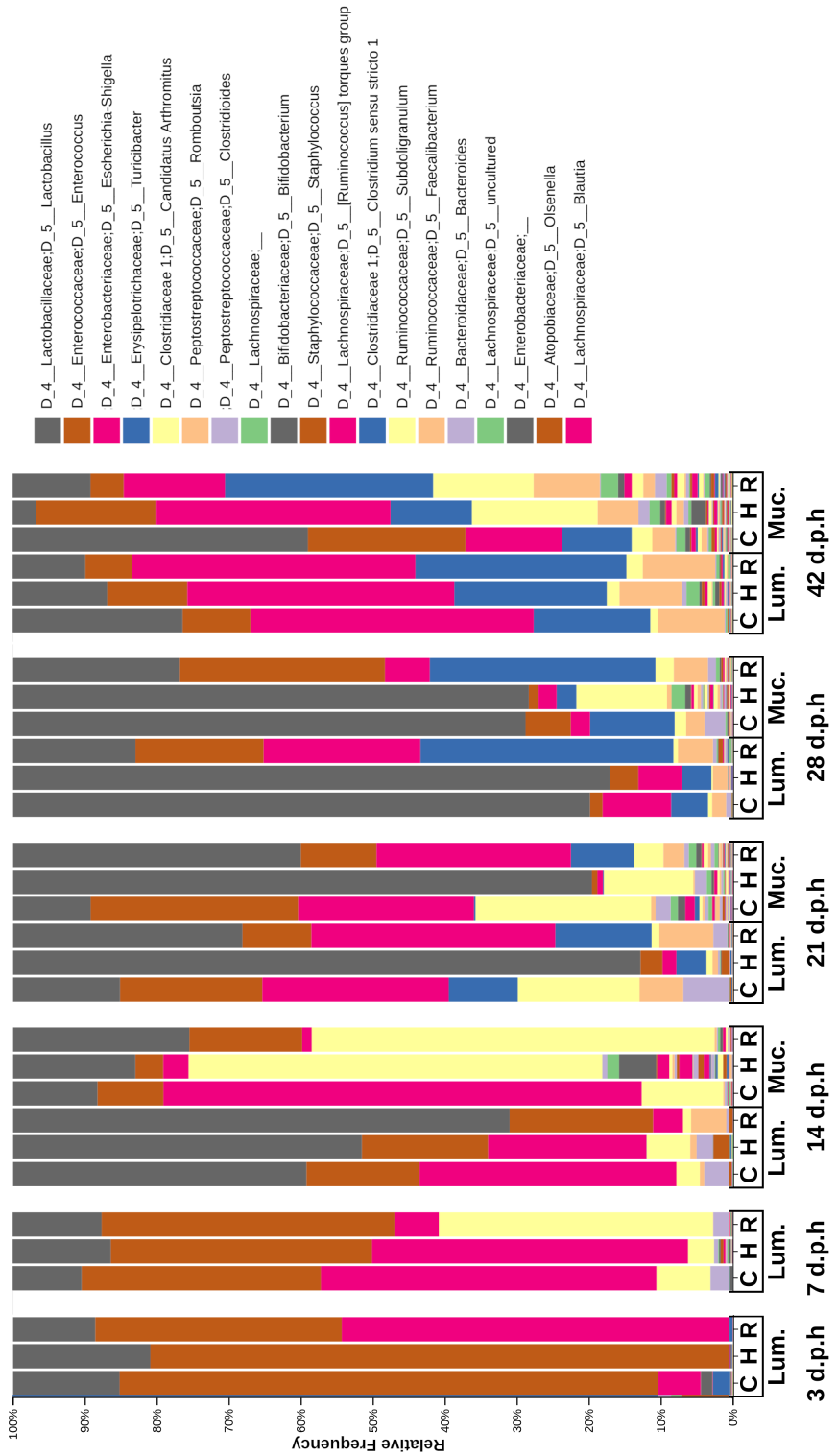


Figure 2.13: Relative abundance of bacterial genera in the ileal mucus and lumen between 3 and 42 d.p.h Lumen (Lum.); Mucus (Muc.); Cobb (C); Hubbard (H); Ross (R)

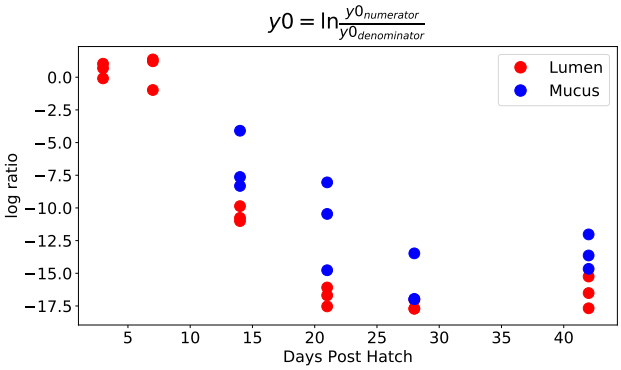
at 14 d.p.h (β = 7.2, $p < 0.001$), allowing $y0_{denominator}$ ASVs to be separated into those that were present at 14 d.p.h ($y1_{numerator}$) and those present from 21 d.p.h ($y1_{denominator}$, Figure 2.14b).

Balance y2 is a subdivision of $y0_{numerator}$ ASVs. The log ratio of balance y2 was significantly higher at 7 (β= 13.2, $p < 0.001$), 14 (β = 17.2, $p < 0.001$), 21 (β = 15.3, $p < 0.001$), 28 (β = 17.6, $p < 0.001$) and 42 (β = 21.1, $p < 0.001$) d.p.h (Figure 2.14c). The dendrogram heatmap (Figure 2.15) shows that this was due to an increased relative abundance of $y2_{denominator}$ ASVs at 3 d.p.h and increasing relative abundance of $y2_{numerator}$ ASVs at later time points.

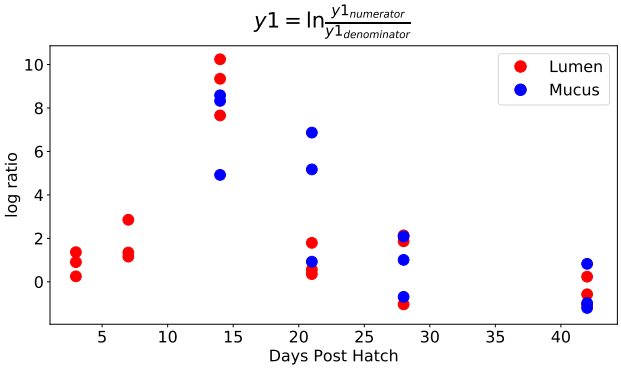
Balance y6 is a subdivision of $y2_{numerator}$ ASVs. The log ratio of balance y6 was significantly lower at 42 d.p.h (β = -12.2, $p = 0.002$) with the dendrogram heatmap showing this was due to an increased relative abundance of $y6_{denominator}$ ASVs at this time point (Figure 2.14d). Balance y10 is a subdivision of $y6_{denominator}$ ASVs and had a significantly lower log ratio at 14 (β = -5.4, $p = 0.02$), 21 (β = -6.0, $p = 0.01$) and 28 (β = -6.3, $p = 0.008$) d.p.h. The dendrogram heatmap shows the lower log ratio was produced by an increased relative abundance of $y10_{denominator}$ ASVs at those time points.

Balance y11 is a subdivision of $y6_{numerator}$ ASVs. The log ratio of balance y11 was significantly lower at 42 d.p.h (β = -9.4, $p = 0.002$) (Figure 2.14f). The dendrogram heatmap shows that $y11_{denominator}$ ASVs were more abundant at 42 d.p.h although some were also present at earlier time points. Balance y18 is a subdivision of $y11_{numerator}$ ASVs. The log ratio of balance y18 was significantly lower at 7 (β = -13.8, $p < 0.001$), 14 (β = -11.5, $p < 0.001$), 21 (β = -12.2, $p < 0.001$), 28 (β = -9.7, $p < 0.001$) and 42 (β = -9.8, $p < 0.001$) d.p.h (Figure 2.14g). The dendrogram heatmap shows that this lower value was caused by a higher relative abundance of $y18_{denominator}$ ASVs. The relative abundance of $y18_{numerator}$ ASVs was sporadic and not related to any time point in particular.

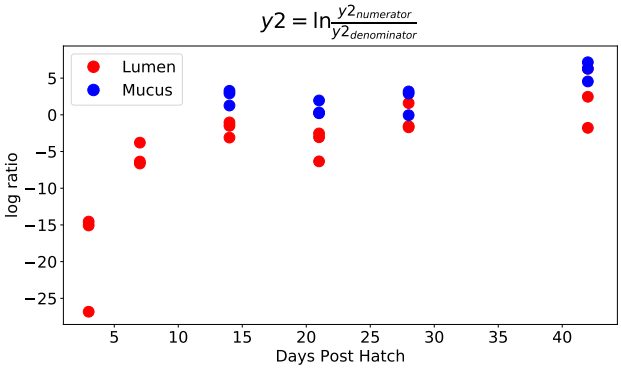
Taxonomic classification of ASVs identified by balances as differentially abundant at different timepoints is presented in Table 2.6. The results of Gneiss analysis mirror what was described by the taxa plots, however, some more details can be discerned. ASVs assigned to Lactobacillaceae were identified as differentially abundant at 3 d.p.h, 14 d.p.h



(a) Balance y0

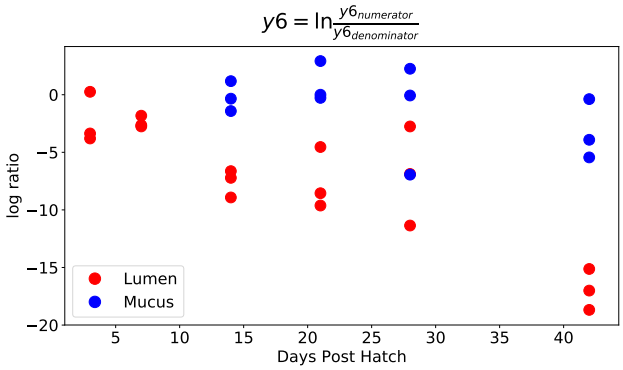


(b) Balance y1

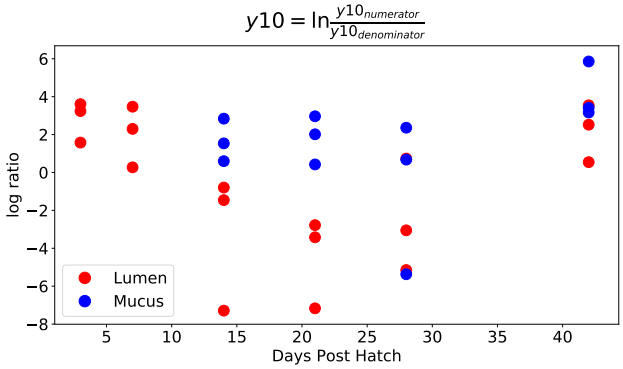


(c) Balance y2

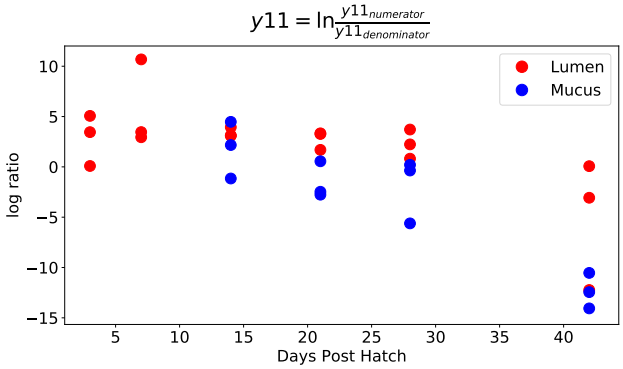
Figure 2.14: Log ratios of balances significantly different between time points during ileal microbiome development



(d) Balance y6

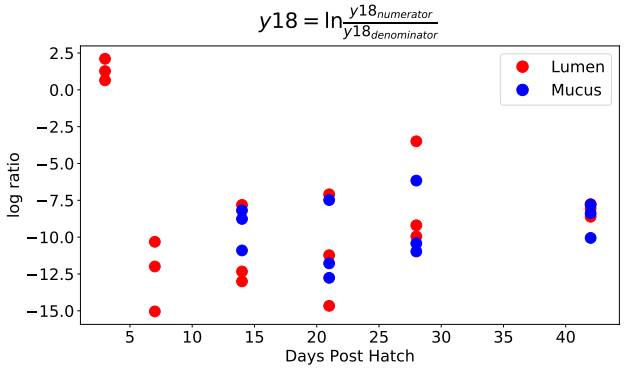


(e) Balance y10



(f) Balance y11

Figure 2.14: Log ratios of balances significantly different between time points during ileal microbiome development



(g) Balance y18

Figure 2.14: Log ratios of balances significantly different between time points during ileal microbiome development

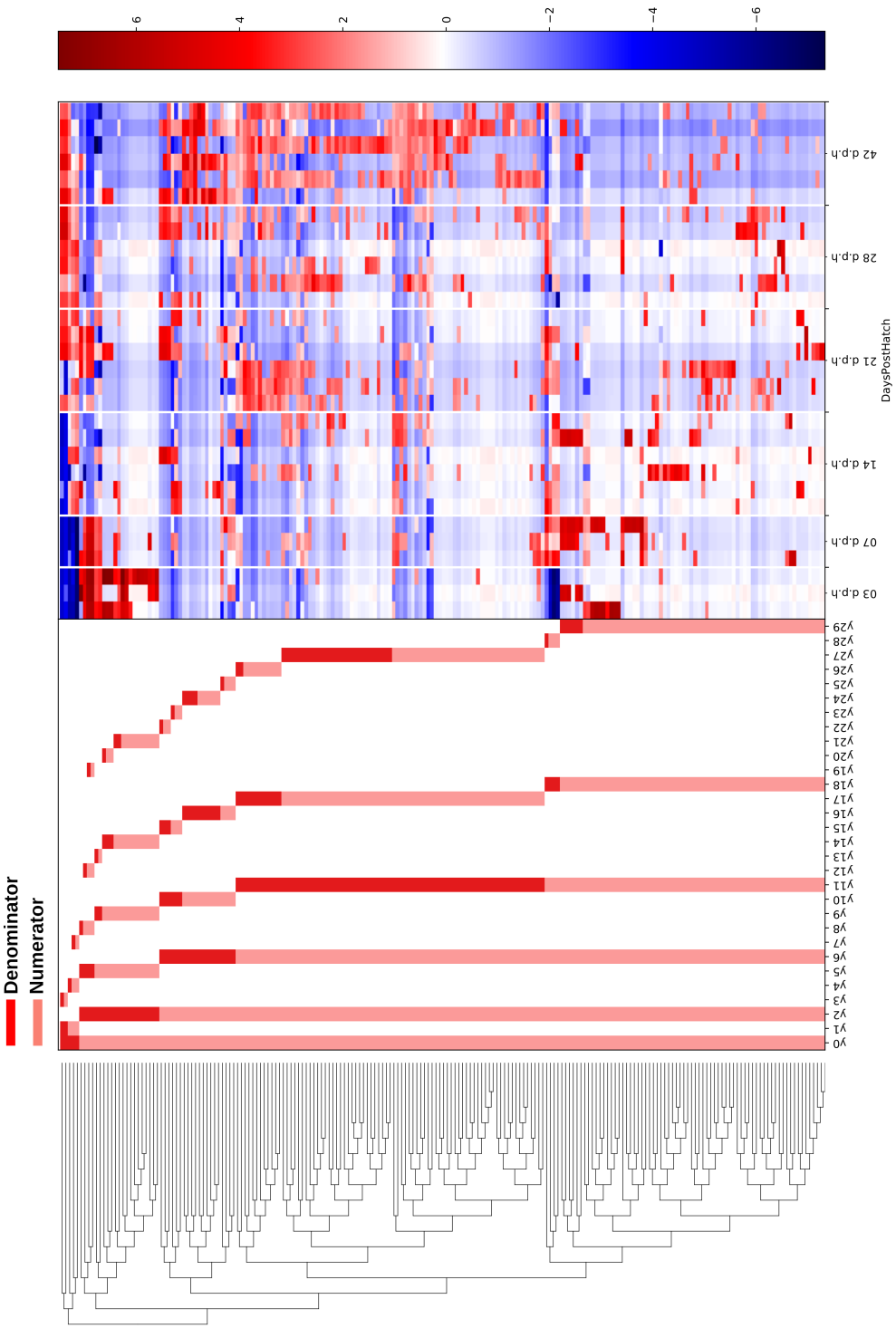


Figure 2.15: Dendrogram heatmap of ASV abundance showing differences in ileal microbiome composition between 3 and 42 d.p.h

A dendrogram heatmap showing the log abundance of ASVs in ileal lumen and mucus samples grouped by age. Differences in relative abundance between the time points are visible in balances y0, y1, y2, y6, y10, y11 and y18 identified by Gneiss analysis as containing differentially abundant ASVs.

and 42 d.p.h. At the species level, Lactobacillaceae ASVs present at 3 d.p.h were classified as *Lactobacillus mucosae* whilst those from later time points were assigned to *Lactobacillus vaginalis* or were not assigned taxonomy further than the genus *Lactobacillus*. ASVs assigned to Erysipelotrichaceae were identified as differentially abundant at 0, 28 and 42 d.p.h. At lower taxonomic levels, those differentially abundant at 0 d.p.h were assigned to *Erysipelatoclostridium* while those at 28 and 42 d.p.h were assigned to *Turicibacter*. Another family which was identified as differentially abundant at multiple time points was Peptostreptococcaceae, with two ASVs abundant at 7 d.p.h, four at 14 d.p.h and two at 42 d.p.h. As with Lactobacillaceae, greater taxonomic resolution revealed that Peptostreptococcaceae ASVs colonising at 7 d.p.h were assigned to the genus *Clostridioides* while later colonisers were assigned to *Romboutsia*. ASVs assigned to Clostridiaceae 1 at 7 d.p.h were further classified as *Candidatus* Arthromitusbut those identified as colonising at 3 and 42 d.p.h were assigned to *Clostridium sensu stricto* 1 at the genus level.

2.6.2 Differences between the lumen and mucus microbiomes

Samples taken between 14 and 42 d.p.h were used to analyse differences between mucus and lumen microbiomes. As before, sampling depth was set at 16,000 for diversity analyses.

Alpha diversity

When alpha diversity was measured using a FPD index, mucus samples had a significantly higher alpha diversity than lumen samples ($H = 11.0$, $p < 0.001$). There were no significant differences in alpha diversity between lumen and mucus samples taken at the same time, however, a plot of FPD alpha diversity shows that mucus samples tended to have a higher alpha diversity than lumen samples (Figure 2.11a). There was no overall effect of area sampled on alpha diversity when measured using a SD index ($H = 1.67$, $p = 0.2$). In this case, alpha diversity plots showed no differences between mucus and lumen samples at 14 and 21 d.p.h, although mucus samples had a higher average alpha diversity at 28 and 42 d.p.h (Figure 2.11b). This indicates that the differences in FPD index were largely driven by low abundance ASVs.

		Number of ASVs					
Taxonomy	**Total Count**	**3 d.p.h**	**7 d.p.h**	**14 d.p.h**	**21 d.p.h**	**28 & 42 d.p.h**	**NDA** [a]
Lachnospiraceae	54	1	0	0	0	39	14
Ruminococcaceae	40	0	0	0	0	28	12
Enterococcaceae	23	13	0	0	0	1	9
Enterobacteriaceae	16	2	0	0	0	4	10
Peptostreptococcaceae	12	0	2	4	0	2	4
Lactobacillaceae	10	2	0	2	0	4	2
Clostridiaceae 1	9	1	2	0	0	2	4
Corynebacteriaceae	4	0	0	1	0	0	3
Staphylococcaceae	4	0	0	2	0	0	2
Erysipelotrichaceae	3	0	0	0	2	1	0
Peptococcaceae	3	0	0	0	0	3	0
Christensenellaceae	2	0	0	0	0	0	2
Aerococcaceae	2	0	0	0	0	1	1
Burkholderiaceae	2	0	0	0	0	1	1
Bacteroidaceae	2	0	0	0	0	2	0
Streptococcaceae	2	1	0	0	0	1	0
Bacillaceae	2	1	0	0	0	1	0
Campylobacteraceae	2	0	0	0	0	1	1
Dermabacteraceae	2	0	0	0	0	0	2
Planococcaceae	1	0	0	0	0	1	0
Pseudomonadaceae	1	0	0	0	0	0	1
Atopobiaceae	1	0	0	0	0	1	0
Bifidobacteriaceae	1	0	0	0	0	0	1
Rhizobiaceae	1	0	0	0	0	0	1
Coriobacteriaceae	1	0	0	0	0	1	0
Paenibacillaceae	1	0	0	0	0	1	0

[a] ASVs defined as NDA were not assigned a time point at which colonisation was significant. Individual taxonomies of significant Gneiss balances are provided in Table A.3.

Table 2.6: Taxonomy of ASVs colonising the ileum at different time points

Beta diversity

When measured using an unweighted UniFrac metric, there was a significant impact of area sampled on beta diversity ($R = 0.63$, $p = 0.001$). However, a significant effect of area sampled was not observed when a weighted UniFrac metric was used ($R = 0.05$, $p = 0.16$). A PCoA plot of unweighted UniFrac beta diversity shows that mucus samples cluster apart from lumen samples (Figure 2.12a) with no clustering pattern visible in the PCoA plot of weighted UniFrac beta diversity (Figure 2.12b). These results suggest that while the composition of the mucus microbiome is different, the relative abundance of the most common ASVs is similar between the mucus and lumen.

Differentially abundant ASVs

Gneiss analysis revealed differential ASV abundance between lumen and mucus samples in the ileum. The ASV table was filtered to exclude ASVs with a frequency of less than 29 reducing the number of ASVs in the analysis from 343 to 178. The overall linear regression model fit was R2 = 0.37 with covariate 'Area' accounting for 14.7% of variance. The log ratio of balances y2 (β = -9.6, p < 0.001), y7 (β = 5.2, p = 0.004), y8 (β = 4.9, p = 0.03), y26 (β = -3.9, p = 0.04) and y30 (β = 3.4, p = 0.04) were significant predictors for 'Area'.

The log ratio of balance y0 was not significantly affected by 'Area' allowing the conclusion that $y0_{denominator}$ ASVs were not differentially abundant between mucus and lumen samples. Balance y2 is a subdivision of $y0_{numerator}$ ASVs and was significantly lower in mucus samples (Figure 2.16a). The dendrogram heatmap shows that this was due to an increased relative abundance of $y2_{denominator}$ ASVs in mucus samples (Figure 2.17).

Balance y4 is a subdivision of $y2_{numerator}$ ASVs but the log ratio of this balance was not significantly different between mucus and lumen samples. Balance y7 is a subdivision of $y4_{denominator}$ ASVs and was significantly lower in lumen samples (Figure 2.16b). The dendrogram heatmap shows that this was due to an increased relative abundance of $y7_{denominator}$ ASVs in lumen samples while $y7_{numerator}$ ASVs were similarly abundant in mucus and lumen samples. Balance y8 is a subdivision of $y4_{numerator}$ ASVs and was significantly lower in lumen samples (Figure 2.16c). The dendrogram heatmap shows that this was due to an increased relative abundance of $y8_{denominator}$ ASVs in lumen samples while $y8_{numerator}$ ASVs were similarly abundant in mucus and lumen samples.

A summary of taxonomic classification of ASVs grouped by higher abundance in the mucus, lumen or NDA is presented in Table 2.7. Most ASVs showed no preference for colonising either the mucus or the lumen. Of the ASVs identified as more abundant in the mucus, the majority were classified as Lachnospiraceae or Ruminococcaceae. Three ASVs assigned to Clostridiaceae 1 were more abundant in mucus. All three of these ASVs were assigned to *Candidatus* Arthromitusat the genus level. The remaining four Clostridiaceae 1 ASVs included in the analysis were classified as not differentially abundant

between mucus and lumen samples and were all assigned to *Clostridium sensu stricto* 1 at the genus level. Some taxa which were previously identified as more abundant in the caecum had a higher relative abundance in the mucus including Peptococcaceae, Bacteroidaceae, Burkholderiaceae, Christensenellaceae and Bacillaceae. Nine ASVs assigned to Lactobacillaceae were included in the analysis. Of these, one was assigned to *Lactococcus*, two to *Lactobacillus mucosae*, three to *Lactobacillus vaginalis* and four to *Lactobacillus*. Both ASVs assigned to *Lactobacillus mucosae* were classified as more abundant in the lumen. 12 ASVs assigned to Peptostreptococcaceae were included in the analysis. Of these, six were assigned to *Romboutsia* and six to *Clostridioides* at the genus level. A total of five ASVs assigned to Peptostreptococcaceae were classified as more abundant in the lumen. At the genus level, four of these ASVs were assigned to *Romboutsia* with the remaining ASV assigned to *Clostridioides*.

2.6.3 Discussion

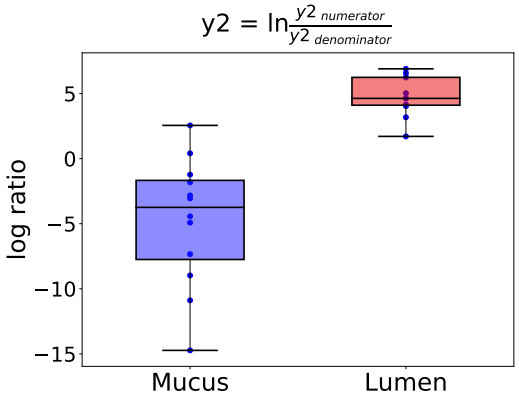
General pattern of succession in the ileal microbiome

The results presented above describe a pattern of succession in the ileal microbiome undergoing several shifts in taxonomic composition before a mature community is present. Most studies observing bacterial succession in the ileum produce differing results. Early microbiomes are the most variable between studies. This is likely due to different bacterial exposure between hatcheries (Pedroso *et al.*, 2005). Most agree that this initial community is replaced by a rise in the abundance of Lactobacillaceae (Park *et al.*, 2015; Ranjitkar *et al.*, 2016; Schokker *et al.*, 2015) however, the timing of this rise often differs between studies. Many factors are likely to contribute to these discrepancies such as environmental exposure, diet and differences in methodology. Stanley *et al.* (2013*b*) showed significant differences in intestinal microbiota between three trials even though the chickens were kept under the same conditions and fed the same diet. There is also no agreement as to whether Lactobacillaceae remains the dominant taxa within the microbiome. Some have found no difference in abundance at time points from 8 to 36 d.p.h while others have observed a

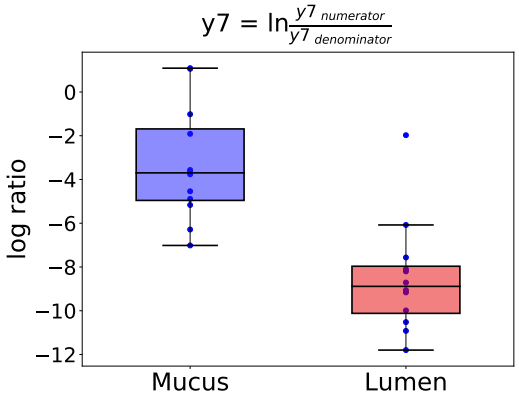
		Number of ASVs		
Taxonomy	**Total**	**Lumen**	**Mucus**	**NDA**[a]
Lachnospiraceae	53	0	18	35
Ruminococcaceae	40	0	23	17
Enterobacteriaceae	14	2	0	12
Peptostreptococcaceae	12	5	0	7
Lactobacillaceae	9	2	1	6
Enterococcaceae	9	4	0	5
Clostridiaceae 1	7	0	3	4
Corynebacteriaceae	4	1	0	3
Staphylococcaceae	4	2	0	2
Erysipelotrichaceae	3	0	0	3
Peptococcaceae	3	0	2	1
Bacteroidaceae	2	0	2	0
Burkholderiaceae	2	0	1	1
Christensenellaceae	2	0	2	0
Campylobacteraceae	2	0	1	1
Streptococcaceae	2	0	0	2
Dermabacteraceae	2	0	0	2
Aerococcaceae	2	0	0	2
Paenibacillaceae	1	0	0	1
Planococcaceae	1	0	0	1
Atopobiaceae	1	0	0	1
Bifidobacteriaceae	1	0	0	1
Coriobacteriaceae	1	0	0	1
Bacillaceae	1	0	1	0

[a] ASVs defined as NDA were not differentially abundant between ileal mucus and lumen samples. Individual taxonomies of significant Gneiss balances are provided in Table A.4.

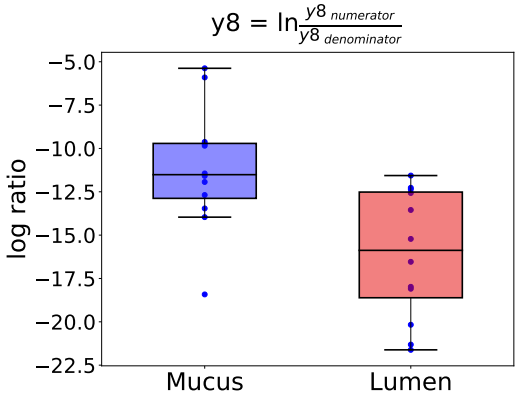
Table 2.7: Taxonomy of differentially abundant ASVs between ileal mucus and lumen samples



(a) Balance y2



(b) Balance y7



(c) Balance y8

Figure 2.16: Log ratios of balances significantly different between ileal lumen and mucus samples

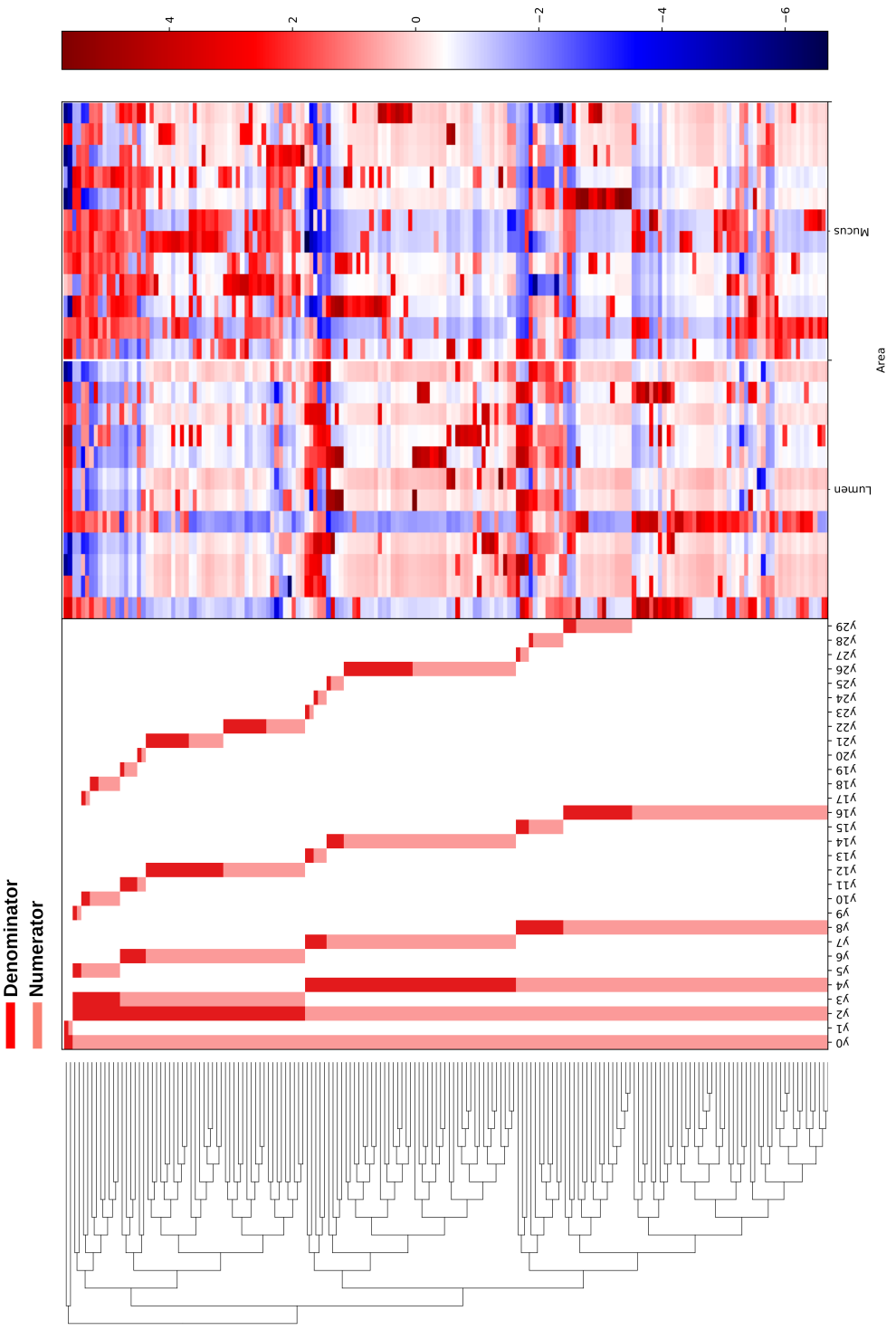


Figure 2.17: Dendrogram heatmap of ASV abundance showing differences in ileal microbiome composition between mucus and lumen samples

A dendrogram heatmap showing the log abundance of ASVs in ileal lumen and mucus samples grouped by age. Differences in relative abundance are visible between the time points in balances $y2$, $y7$, $y8$ identified by Gneiss analysis as containing differentially abundant ASVs.

decrease in Lactobacillaceae between 4 and 16 d.p.h (Lumpkins *et al.*, 2010; Schokker *et al.*, 2015).

A similar pattern of succession to that described in this study has been proposed by Jurburg *et al.* (2019) after examining the faecal microbiome of chickens between 1 and 35 d.p.h although many taxa detected were associated with the caecal microbiome rather than the ileal microbiome. The first stage was dominated by rapidly-colonising taxa such as *Streptococcus* and *Escherichia/Shigella*. *Lactobacillus* became more prominent in the faecal microbiome from 3 d.p.h with an increase in relative abundance at 14 d.p.h. The peak of *Candidatus* Arthromitusabundance was noted at 14 and 21 d.p.h. Slower growing taxa such as *Romboutsia* and other Peptostreptococcaceae colonised from 21 d.p.h. The similarities between these results and those described above support the conclusion that the ileal microbiome undergoes several shifts in composition from hatch to 35 and 42 d.p.h. Further evidence that the ileal microbiome isn't stable until later time points can be found in the results of those using techniques other than NGS. Lu *et al.* (2003), found that clone libraries at 3, 7 and 49 d.p.h had a high dissimilarity to those from 14, 21 and 28 d.p.h with *Lactobacillus* decreasing while *Clostridia* increased over time. Similarly, den Hartog *et al.* (2016) used T-RFLP reads to demonstrate a temporary disturbance in ileal microbiome composition between 14 and 42 d.p.h.

I am inclined to conclude that high Lactobacillaceae abundance is not the hallmark of a mature ileal microbiome but rather a stage of maturation. Much work has been done to characterise the bacterial composition of the ileal microbiome in chickens at fixed time points. Many of these focus on the microbiome between 21 and 35 d.p.h, presumably under the impression that the ileal microbiome has matured by this point. Indeed, Amit-Romach *et al.* (2004) stated that "A typical microflora of adult birds in the small intestine is established within 2 weeks". This has perhaps led to the assumption that a mature ileal microbiome is dominated by Lactobacillaceae.

Although the general pattern of succession followed that described by previous studies, examination of Lactobacillaceae and Peptostreptococcaceae ASVs at the genus and species level revealed a more detailed pattern of succession. Most importantly, ASVs assigned to

Lactobacillus mucosae colonised the ileum earlier than those assigned to other species of Lactobacillaceae. Although the order of succession in the ileal microbiome may simply be a matter of presence or absence of taxa it is possible that some strains of bacteria are better adapted to colonise the early gut whilst others require alterations to the gut environment produced by early inhabitants before they are able to colonise. While more detailed studies considering bacterial succession using identification at higher taxonomic levels would be required to confirm this hypothesis, it is worth considering in terms of selection of potential probiotic species. Currently, probiotics may be selected due to *in vitro* activity against pathogens, production of certain metabolites or suitability to industrial production and administration. However, the ability of strains to colonise the gut immediately post-hatch would also influence the functional success of a probiotic product.

This study should also highlight two groups of ileal bacteria that have previously been neglected but which may be of interest. Firstly, the ASVs assigned to Peptostreptococcaceae in this study were further identified as *Clostridioides* and *Romboutsia* with *Clostridioides* colonising the ileum from 7 d.p.h and *Romboutsia* present from 14 d.p.h. The human pathogen *Clostridium difficile* was reclassified in 2016 to the genus *Clostridioides* (Lawson *et al.*, 2016). The two ASVs assigned to *Clostridioides* in this study were not classified to the species level by comparison against the SILVA database. A search of the NCBI bacterial 16S rRNA gene database using BLAST revealed 98.13 and 98.51% similarity to sequences from *Clostridioides difficile*. The presence of *Clostridioides difficile* in the chicken ileum has been reported and discussed with respect to public health (Candel-Pérez *et al.*, 2019) but no studies mention the impact of this species on poultry health. Genomic and functional analysis of *Romboutsia* found in the human gastrointestinal tract reveal that these Clostridia are highly adapted to life in the small intestine with the ability to ferment glucose and other simple carbohydrates (Gerritsen *et al.*, 2017). Secondly, Erysipelotrichaceae was found in the ileum from 21 d.p.h in this study. These ASVs were classified as *Turicibacter* at the genus level. Studies of *Turicibacter* metabolism and functional genomics are not yet available, however, the abundance of *Turicibacter* in this study should highlight it as another neglected but important member of the mature ileal microbiome.

Differences in succession between breeds

When describing differences in succession between breeds using the results described above, the limitations of sample size and pooling should be considered. Small sample size reduces the ability to support observations with statistical tests. As such, the description of differences in succession between breeds is purely observational and without statistical support. Equally, pooling samples masks individual variation between chickens. It is possible that pooled samples were more representative of certain constituent samples than others.

The general pattern of succession described above was followed by all breeds with the exception that no sudden rise in *Candidatus* Arthromitusabundance was observed in Cobb chickens. However, the rate of succession differed between breeds. Ross birds were the first to demonstrate sequential rises in Enterobacteriaceae (3 d.p.h), *Candidatus* Arthromitus(7 d.p.h), Lactobacillaceae (14 d.p.h) and Erysipelotrichaceae (28 d.p.h). This pattern was followed, first by Hubbard chickens and then by Cobb chickens. The peak in *Candidatus* Arthromitusabundance was visible in Hubbard birds at 14 d.p.h and may have occurred in Cobb birds between 14 and 21 d.p.h. Lactobacillaceae abundance rose in Hubbard birds at 21 d.p.h and Cobb birds at 28 d.p.h. The dominance of Lactobacillaceae was more sustained in Hubbard birds as it was still observable seven days later at 28 d.p.h. An increase in Enterobacteriaceae and Erysipelotrichaceae with a concurrent decrease in Lactobacillaceae was apparent in both Cobb birds and Hubbard birds by 42 d.p.h.

Three previous studies provide direct comparisons between Ross, Cobb and Hubbard genotypes. They focus on the responses of the three breeds to necrotic enteritis. In general, they have found that Cobb chickens are more susceptible to necrotic enteritis than Ross and Hubbard chickens, exhibiting increased body weight loss and more severe intestinal lesions (Jang *et al.*, 2013). Hubbard broilers appear to have an intermediary susceptibility to necrotic enteritis with Ross broilers emerging as the most resistant. In one study, Hubbard chicks lost more body weight than Ross chicks but did not have more severe intestinal lesions (Kim *et al.*, 2015). In another, Hubbard chicks showed no significant loss of body

weight compared to Ross chicks and showed an intermediate severity of intestinal lesions between Ross and Cobb chicks (Jang *et al.*, 2013). Ross broilers have been shown to have differential expression of β-defensin genes during necrotic enteritis infection when compared to Cobb broilers (Hong *et al.*, 2012). These results are interesting in light of the observation above that Ross broilers had an accelerated development of the ileal microbiome when compared to Cobb broilers, with Hubbard broilers as an intermediary.

The protocol for inducing necrotic enteritis in all three experiments was the same. *Eimeria maxima* oocyts were administered at 14 d.p.h followed by *Clostridium perfringens* at 18 d.p.h (Hong *et al.*, 2012; Kim *et al.*, 2015; Jang *et al.*, 2013). It follows that any difference in response to necrotic enteritis should have its roots during the first two weeks of life. The most evident difference between the three breeds during this period was the appearance of Candidatus *Arthromitus*. This genus formed a great part of the microbiome in Ross chicks from 7 to 14 d.p.h and Hubbard chicks at 14 d.p.h, but was less abundant in Cobb chicks. It is possible that this lack of early colonisation by *Candidatus* Arthromituscould leave Cobb chickens more susceptible to infectious disease as the interaction between *Candidatus* Arthromitusand host tissue plays a role in immune maturation.

Stanley *et al.* (2014*b*) used Cobb chickens to investigate changes in caecal microbiota to dietary fishmeal, *Eimeria* and *C. perfringens* in a model of necrotic enteritis. The abundance of *Candidatus* Arthromitus was observed to increase in response to infection with *C. perfringens* but was not present in any groups treated with *Eimeria*. The authors suggest that *Eimeria* could remove *Candidatus* Arthromitus as a mechanism for modulating host immunity and thereby increase its own infectivity. However, the removal of *Candidatus* Arthromitus leads to an imbalance in mucosal immunity allowing *C. perfringens* to grow unchecked (Stanley *et al.*, 2014*b*). Further weight is added to this argument by the observation that fumonisin mycotoxins in feed also lower the abundance of *Candidatus* Arthromitus and increase the susceptibility to *C. perfringens* infection and development of necrotic enteritis (Antonissen *et al.*, 2015).

Candidatus Arthromitus: colonisation and immune development

Candidatus Arthromitus is a segmented filamentous bacteria (SFB), a group of host specific, non-pathogenic bacteria which are often found associated with the terminal ileum of animals (Snel *et al.*, 1995). In recent years the appropriate taxonomy has been debated. *Candidatus* Arthromitus was originally proposed due to the morphological similarity to bacteria associated with the arthropod intestinal tract discovered by Joseph Leidy in 1849 (Leidy, 1849; Snel *et al.*, 1995). However, 16S rRNA gene sequencing has revealed that, despite their morphological similarity, Leidy's arthropod SFB belong to Lachnospiraceae whilst those described in vertebrates belong to Clostridiaceae (Thompson *et al.*, 2012). In light of this the name Candidatus *Savagella* has been proposed although both names are still used in the literature (Thompson *et al.*, 2012).

The life cycle of SFB is complex and involves extensive contact with the ileal epithelium but since SFB are unculturable details must be discerned from microscopy (Ericsson *et al.*, 2014). SFB exist in two morphological forms, a dormant spore and an active holdfast. Some studies suggest that the holdfasts begin to grow in the lumen before developing specialised ends which invade the epithelium (Caselli *et al.*, 2010). Others hypothesise that holdfasts are initially motile and attach to the epithelium before more segments begin to grow (Pamp *et al.*, 2012). Each segment develops two intrasegmental bodies which differentiate into either spores or holdfasts which are released into the lumen (Pamp *et al.*, 2012).

Previous studies observing the colonisation and distribution of SFB in the chicken gut have produced similar results to those presented above. SFB are found principally in the ileal mucus with some presence in ileal content (Liao *et al.*, 2012) although there is one report of SFB in the caecal tonsils (Glick *et al.*, 1978). SFB appear during the first week of life, reaching peak abundance between 9 and 14 d.p.h before declining (Glick *et al.*, 1978; Liao *et al.*, 2012). The pattern of colonisation varies greatly among individual birds, especially at an early age. One possible explanation is the influence of other members of the microbiome. Earlier SFB colonisation was observed in chickens fed a *Lactobacillus delbrueckii* probiotic (Liao *et al.*, 2012). Equally this variation could be explained by

differing levels of maternal IgA transfer to chicks. The decline of SFB in chicks is linked to increasing concentrations of intestinal IgA (Liao *et al.*, 2012; Suzuki *et al.*, 2004). This is similar to observations in mice in which SFB colonisation doesn't occur until weaning when maternal IgA is no longer supplied to the gut with the population of SFB declining again once endogenous IgA production reaches sufficient levels (Jiang *et al.*, 2001).

All studies investigating the effect of SFB on host immune development have used comparisons between gnotobiotic and germfree mice. Any comparison between mammalian and avian immune development is likely to produce false assumptions not only because SFB appear to interact with different epithelial cells between species (Ericsson *et al.*, 2014) but also because the function of some immune cell subsets in avian immunology is still unknown. However, it is worth noting that SFB have been shown to stimulate various parts of the mammalian immune system and it would be worth pursuing similar experiments in chickens to discern the role that SFB play in the development of the avian immune system. SFB have been linked to such diverse roles as the stimulation of IgA production (Klaasen *et al.*, 1993; Talham *et al.*, 1999; Umesaki *et al.*, 1999), increasing numbers of αβ– and αα– intraepithelial T-cells (Ivanov *et al.*, 2009; Umesaki *et al.*, 1995), inducing expression of fucosyl sialo GM1 glycolipids which may inhibit attachment of other bacteria (Umesaki *et al.*, 1995), induction of Th17 cells and decreased regulatory T cells (Ivanov *et al.*, 2009; Ericsson *et al.*, 2014).

Differences between mucus and lumen microbiomes

Significant differences between the composition of the mucus and lumen microbiomes were discovered. *Candidatus* Arthromituswas identified as significantly more abundant in the mucus. This finding is likely due to Candidatus *Arthromitus*' close association with the ileal epithelium during its life cycle (Ericsson *et al.*, 2014). Other taxa identified as differentially abundant in the mucus were those previously classified as associated with the caecal microbiome such as Lachnospiraceae, Ruminococcaceae, Peptococcaceae and Bacteroidaceae. A similar result was obtained by Borda-Molina *et al.* (2016) who found Lachnospiraceae, Ruminococcaceae and Burkholderiaceae to be more prevalent in the crop

and ileal mucosa compared to digesta. Their presence in the ileum may be due to ingestion of these bacteria from the faeces of other birds or retroperistalsis from the caecum.

Some taxa were identified as differentially abundant in the ileal lumen including *Romboutsia*, two Lactobacillaceae, four Enterococcaceae and two Staphylococcaceae. In the absence of quantitative data it is not possible to discern whether the increase in relative abundance of these ASVs in the lumen was due to an absolute increase in their abundance or a decrease in the abundance of Candidatus *Arthromitus*. If a true biological preference for the lumen were present it would be expected that a majority, if not all, ASVs assigned to a taxa would be differentially abundant in that niche as in the case of Candidatus *Arthromitus*. This was not observed for Lactobacillaceae, Enterococcaceae or Staphylococcaceae. Four of six ASVs assigned to *Romboutsia* were differentially abundant in the lumen, presenting a stronger case for a true biological preference for lumen colonisation. Under laboratory conditions, *Romboutsia* is unable to grow on mucin which is likely to due its inability to degrade mucus derived carbohydrates such as L-fucose and sialic acid (Gerritsen *et al.*, 2017). This experimental data supports the finding that *Romboutsia* was differentially abundant in the lumen and may have a higher absolute abundance in the lumen.

2.6.4 Summary

The early ileal microbiome had a low diversity with Enterobacteriaceae and Enterococcaceae found to be the most abundant taxa. *Lactobacillus mucosae* was present at 3 d.p.h but other species of *Lactobacillus* such as *Lactobacillus vaginalis* did not colonise the ileum until later time points. A pattern of succession followed with *Candidatus* Arthromitus and *Clostridioides* appearing in the ileal microbiome at 7 d.p.h. *Candidatus* Arthromitus became the most abundant taxa in the mucus while Lactobacillaceae was the most abundant in the lumen. The high abundance of *Candidatus* Arthromitus was short lived as Lactobacillaceae became the most abundant genus in both the mucus and lumen. High abundance of Lactobacillaceae was a transient feature of the ileal microbiome with Peptostreptococcaceae, Enterobacteriaceae, Enterococcaceae and Erysipelotrichaceae increasing in abundance at later time points. This general pattern of succession was followed by all breeds, however, the

rate at which succession occurred was different with the intestinal microbiome of Ross birds advancing through the described pattern of succession quicker than Hubbard and Cobb birds. These differences in succession, especially a disparity in *Candidatus* Arthromitus abundance, could explain differences in the susceptibility to infectious enteric disease previously observed between these three breeds.

Significant differences between the lumen and mucus microbiomes were observed with *Candidatus* Arthromitus and caecal bacteria such as Lachnospiraceae, Ruminococacaeae and Burkholderiaceae showing increased abundance in the mucus and *Romboutsia* showing increased abundance in the lumen.

2.7 The Caecal Microbiome

The development of the caecal microbiome between 0 and 42 d.p.h was observed in the three breeds of chicken. Lumen samples were taken from 0 d.p.h and mucus samples from 14 d.p.h. Sampling depth was set at 23,000 for diversity analyses with all samples included in the analysis.

2.7.1 Succession in the caecal microbiome

Alpha diversity

Overall, age had a significant effect on alpha diversity when measured using both FPD index and SD index ($H = 29.9$, $p < 0.001$ and $H = 21.0$, $p = 0.002$ respectively) with an increase in alpha diversity over time (Figure 2.18). There were increases in mean FPD and SD indices between 0 and 3 d.p.h and 3 and 7 d.p.h (0 d.p.h = 2.12; 3 d.p.h = 3.82; 7 d.p.h = 5.13, and 0 d.p.h = 2.52; 3 d.p.h = 3.45; 7 d.p.h = 4.10, respectively) although these differences were not significant because of the small sample size. There was a significant increase in FPD and SD between 7 and 14 d.p.h ($H = 5.2$, $p = 0.04$ and $H = 5.4$, $p = 0.04$ respectively). From 14 d.p.h there were no further increases in SD index, however, the FPD index continued to increase with significant increases between 14 and 21 d.p.h ($H = 5.77$, $p = 0.02$), 21 and 28 d.p.h ($H = 5.03$, $p = 0.03$) and 28 and 42 d.p.h ($H = 6.56$, $p = 0.02$).

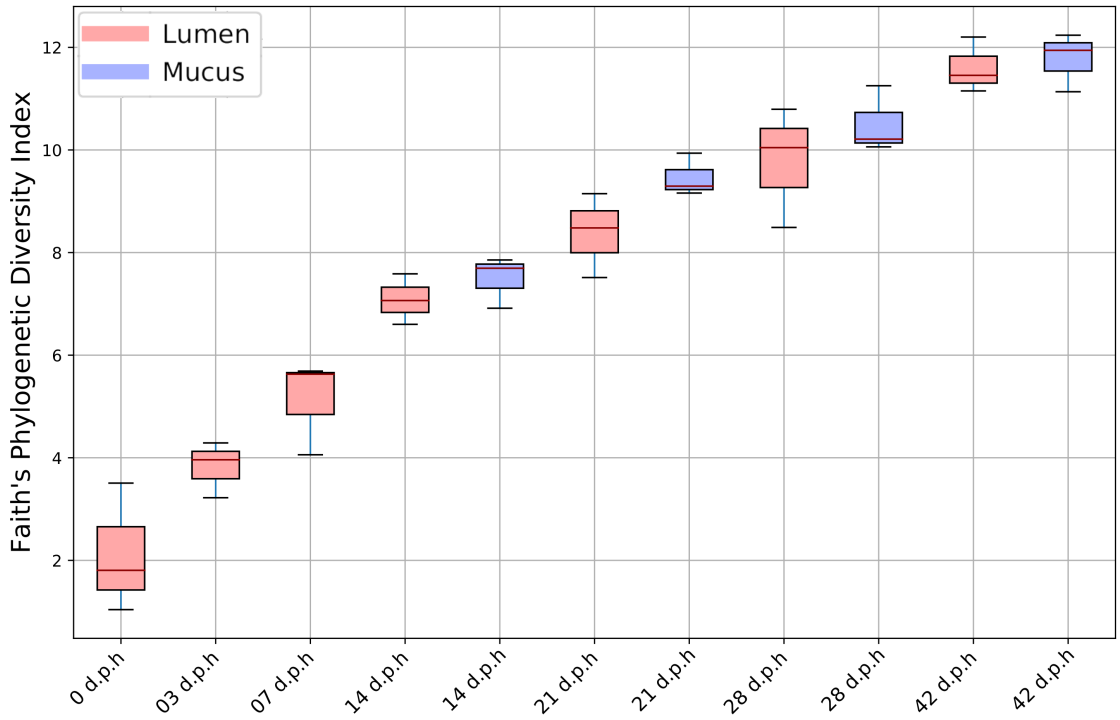
This suggests that while new taxa were joining the caecal microbiome between 14 and 42 d.p.h, there was no corresponding increase in community evenness.

Beta diversity

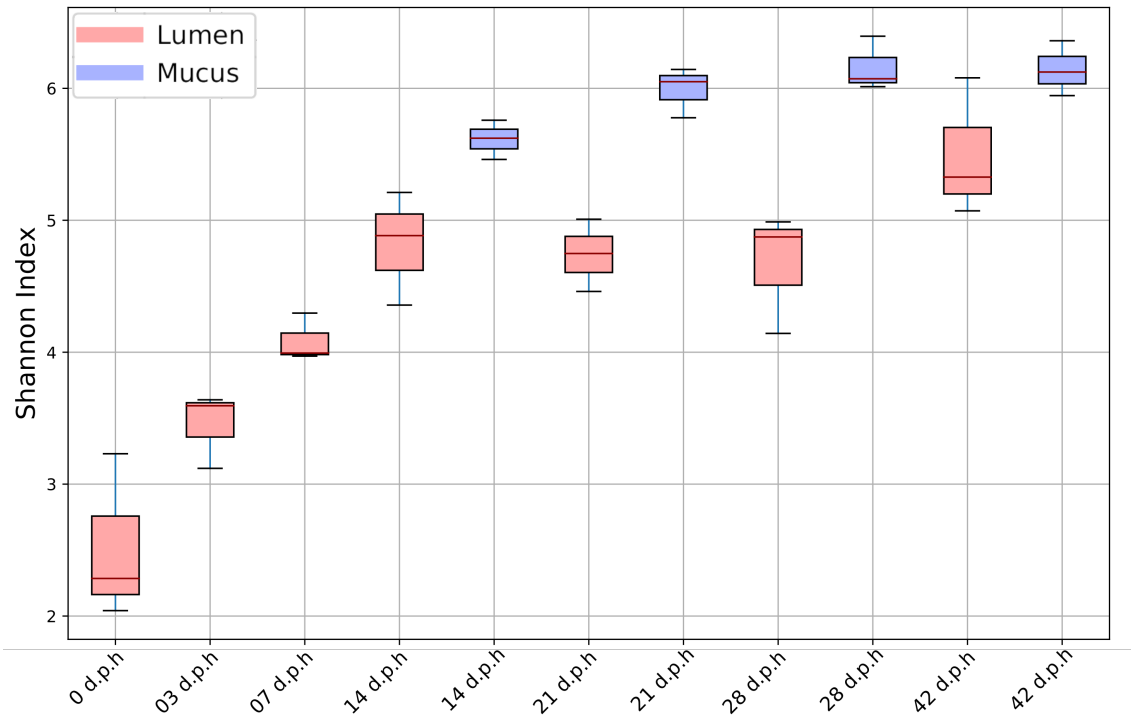
From the β-diversity plots a pattern of succession becomes apparent. Beta diversity was significantly affected by days post hatch when measured using an unweighted (R- statistic = 0.84, p = 0.001) and weighted UniFrac metric (R-statistic = 0.50, p = 0.001). Clustering of samples by age is visible in PCoA plots of both unweighted and weighted UniFrac distances (Figure 2.19). The caecal community at 0 d.p.h was different when compared to other time points with more variation between samples taken at the same time. At 3 d.p.h, samples clustered closer together in both unweighted and weighted UniFrac plots suggesting a more similar composition. Samples from 3 d.p.h tended to be closer to those from 0 d.p.h than later time points. At 7 d.p.h the microbiota was more similar to later time points. In unweighted UniFrac plots, samples continued to form separate clusters by sampling time. However, in the weighted UniFrac plot only samples from 14 d.p.h continued to cluster separately with samples from 21, 28 and 42 d.p.h clustering together. There was also a noticeable separation between mucus and lumen samples from 14 d.p.h in the weighted UniFrac plot (Figure 2.19b). Differences in clustering by unweighted and weighted UniFrac distance suggest that although new taxa continued to join the caecal microbiome until 42 d.p.h, there were no significant changes in the relative abundance of major taxa between 21 and 42 d.p.h. In this respect, the largest alteration in the caecal microbiome occurred between 3 and 7 d.p.h.

Differentially abundant ASVs between time points

Gneiss analysis and taxa plots revealed differential ASV abundance between time points. The feature table was filtered to exclude ASVs with a frequency of less than 102, reducing the number of ASVs included from 847 to 424. For Gneiss analysis, the overall linear regression model fit was R2 = 0.654 with covariate "Days Post Hatch" accounting for 56% of variance. The log ratio of balances y0 (42 d.p.h: β = -31.1, $p < 0.001$; 28 d.p.h: β = -22.9,

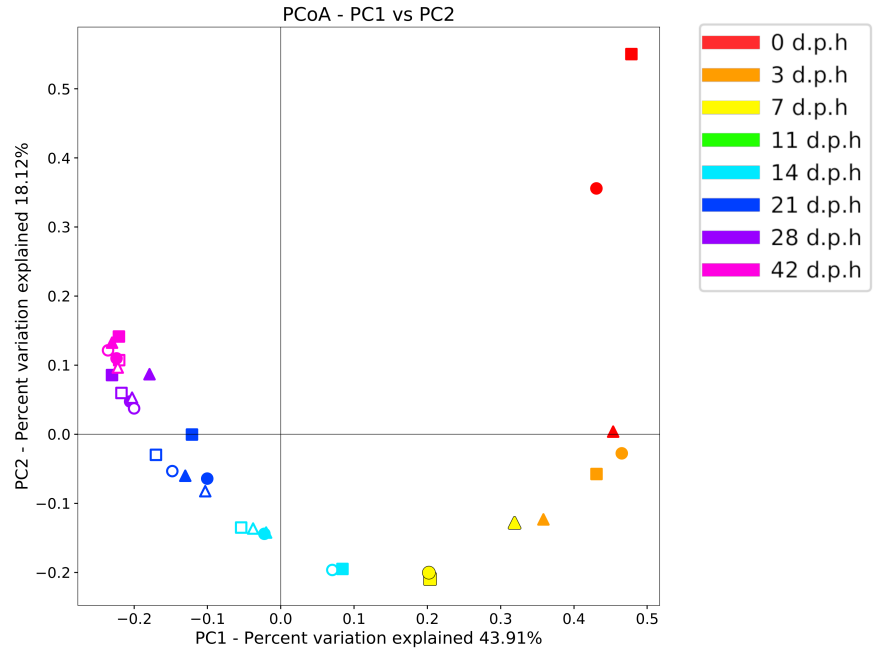


(a) FPD Index

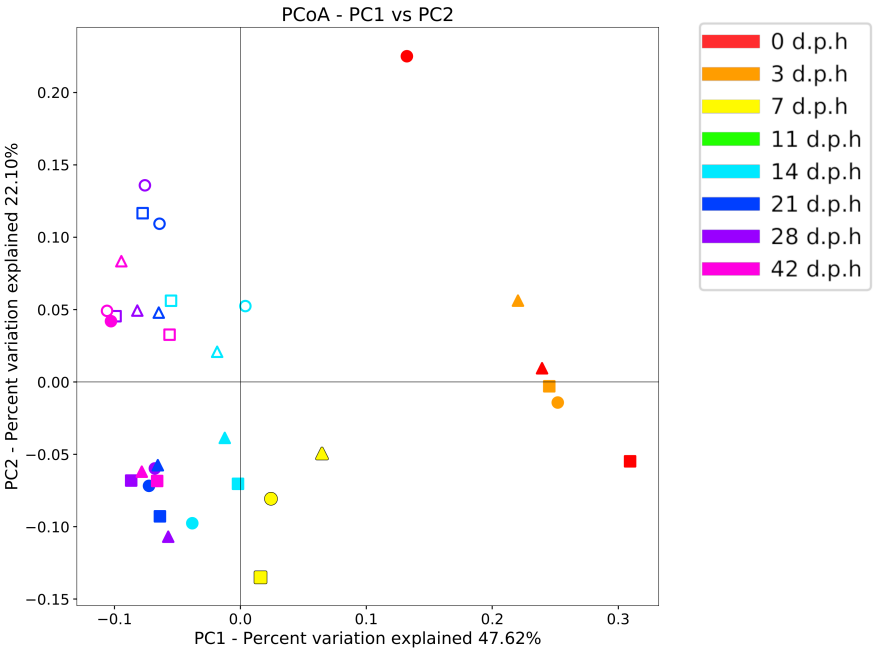


(b) SD Index

Figure 2.18: Alpha diversity in caecal lumen and mucus samples from 0 to 42 d.p.h



(a) Unweighted UniFrac



(b) Weighted UniFrac

Figure 2.19: Beta diversity in the caecum from 0 to 42 d.p.h

Samples from Hubbard (▲), Ross (■) and Cobb (●) chickens are shown with lumen (filled) and mucus (no fill) samples denoted by colour fill.

$p < 0.001$; 21 d.p.h: β = -10.7, p = 0.02), y1 (42 d.p.h: β = -20.4, $p < 0.001$; 28 d.p.h: β = -25.4, $p < 0.001$; 21 d.p.h: β = -25.7.5, $p < 0.001$; 14 d.p.h: β = -21.5, $p < 0.001$; 7 d.p.h: β = -7.1, p = 0.007), y2 (42 d.p.h: β = 21.5, $p < 0.001$; 28 d.p.h: β = 26.2.5, $p < 0.001$; 21 d.p.h: β = 31.1, $p < 0.001$; 14 d.p.h: β = 32.2, $p < 0.001$; 7 d.p.h: β = 19.6, $p < 0.001$), y3 (21 d.p.h: β = -11.6, p = 0.001; 14 d.p.h: β = -20.2, $p < 0.001$; 7 d.p.h: β = -10.7, p = 0.008), y4 (42 d.p.h: β = 15.4, $p < 0.001$; 21 d.p.h: β = -8.0, p = 0.005), y5 (42 d.p.h: β = 14.4, $p < 0.001$; 28 d.p.h: β = 13.2, $p < 0.001$; 21 d.p.h: β = 14.5, $p < 0.001$; 14 d.p.h: β = 16.3, $p < 0.001$; 7 d.p.h: β = 20.2, $p < 0.001$, 3 d.p.h: β = 22.9, $p < 0.001$), y6 (42 d.p.h: β = 8.8, $p < 0.001$; 14 d.p.h: β = 6.5, p = 0.01; 7 d.p.h: β = 21.7, $p < 0.001$, 3 d.p.h: β = 9.6, p = 0.001), y7 (14 d.p.h: β = 5.3, p = 0.006; 7 d.p.h: β = 13.4, $p < 0.001$) and y8 (21 d.p.h: β = 11.9, $p < 0.001$; 14 d.p.h: β = 7.7, p = 0.04) were significantly different at one or more time points. On inspection of the dendrogram heatmap (Figure 2.20) these balances revealed waves of colonisation in the caecal microbiota. Full descriptions of ASV taxonomy by balance are provided in Table A.5.

The log ratio of balance y0 had a high value at 0 d.p.h suggesting that some ASVs in $y0_{numerator}$ were more abundant at this time (Figure 2.21a). Between 3 and 42 d.p.h, the log ratio decreased as there was a shift in relative abundance from $y0_{numerator}$ to $y0_{denominator}$ ASVs. The initial abundance and subsequent decline of $y0_{numerator}$ ASVs can be observed in the dendrogram heatmap (Figure 2.20) and the taxa plot (Figure 2.22). However, examination of further balances is required to fully distinguish ASVs prevalent at 0 d.p.h from those present at 3, 7 and 14 d.p.h.

Balance y2 is a subdivision of $y0_{numerator}$. The log ratio of balance y2 was lower at 0 d.p.h as there was a higher abundance of $y2_{denominator}$ ASVs (Figure 2.21c). There was an increase in log ratio from 7 to 14 d.p.h as the relative abundance of numerator ASVs increased (Figure 2.20). From 21 d.p.h, the log ratio began to decrease as the balance was shifted back towards 0 by a decrease in relative abundance of $y2_{numerator}$ ASVs. It can be concluded that ASVs which colonised the caecum between 7 and 14 d.p.h are represented by $y2_{numerator}$. Further resolution is provided by balance y6 whose log ratio increased from 0 to 7 d.p.h due to an increase in $y6_{numerator}$ ASVs at 3 and 7 d.p.h before declining at 14

d.p.h as $y6_{denominator}$ ASVs began to colonise the caecum (Figure 2.21g).

Balance y5 is a subdivision of $y2_{denominator}$ which allows the distinction between ASVs which colonised at 0 and 3 d.p.h. The log ratio of balance y5 increased from 0 to 3 d.p.h (Figure 2.21f) suggesting that $y5_{denominator}$ ASVs were more abundant at 0 d.p.h with a higher prevalence of $y5_{numerator}$ ASVs at 3 d.p.h. This pattern is confirmed by the dendrogram heatmap (Figure 2.20).

As previously discussed, ASVs associated with later time points are described by $y0_{denominator}$, but further balances are required to discern at which time point ASVs became prevalent. Balance y1 is a subdivision of $y0_{denominator}$. The log ratio of balance y1 was close to 0 at 0 and 3 d.p.h as both numerator and denominator ASVs were absent. There was a slight decrease at 7 d.p.h as some denominator ASVs colonised the caecum. This decrease continued at 14 and 21 d.p.h before the log ratio began to increase again at 42 d.p.h (Figure 2.21b). This suggests that $y1_{denominator}$ ASVs were associated with colonisation between 7 and 21 d.p.h while $y1_{numerator}$ ASVs represent later colonisers. Balance y3 is a subdivision of $y1_{denominator}$. The log ratio of balance y3 was close to 0 at 0 and 3 d.p.h as discussed for balance y1. There was a decrease in log ratio between 7 and 14 d.p.h followed by an increase from 21 d.p.h (Figure 2.21d). This shows that $y3_{denominator}$ ASVs were associated with colonisation between 7 and 14 d.p.h while $y3_{numerator}$ ASVs represent later colonisers. Balance y7 provides further resolution of $y3_{denominator}$ ASVs and shows that $y7_{numerator}$ ASVs were associated with colonisation at 7 d.p.h while $y7_{denominator}$ ASVs began to colonise the caecum from 14 d.p.h (Figure 2.21h). Equally, balance y8 provides further resolution of $y3_{numerator}$ ASVs and shows that $y8_{numerator}$ ASVs colonised the caecum at 14 d.p.h while $y8_{denominator}$ colonised from 21 d.p.h (Figure 2.21i). Finally, balance y4 is a subdivision of $y1_{numerator}$. The log ratio of balance y4 was close to 0 at 0, 3 and 7 d.p.h. There was a slight decrease in log ratio at 14 d.p.h with a further decrease noticeable at 21 d.p.h (Figure 2.21e). The dendrogram heatmap shows that this decrease in log ratio can be attributed to an increase in the relative abundance of $y4_{denominator}$ ASVs. There was an increase in log ratio at 28 and 42 d.p.h attributable to an increase in the relative abundance of $y4_{numerator}$ ASVs.

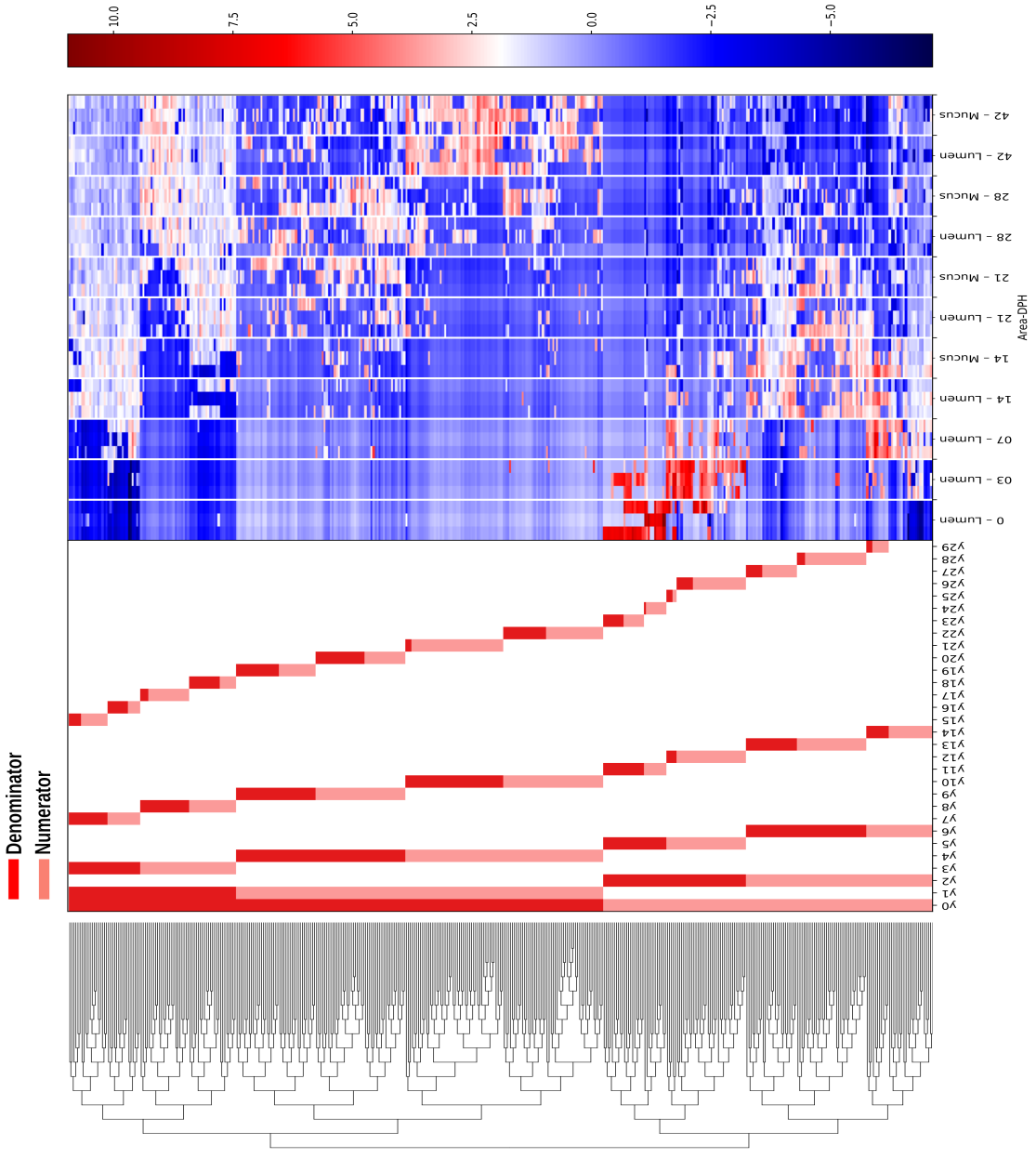
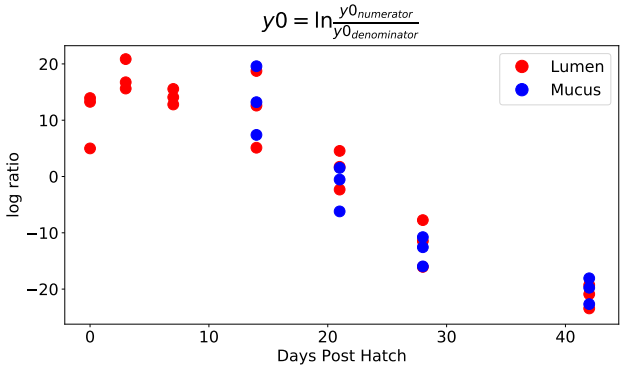
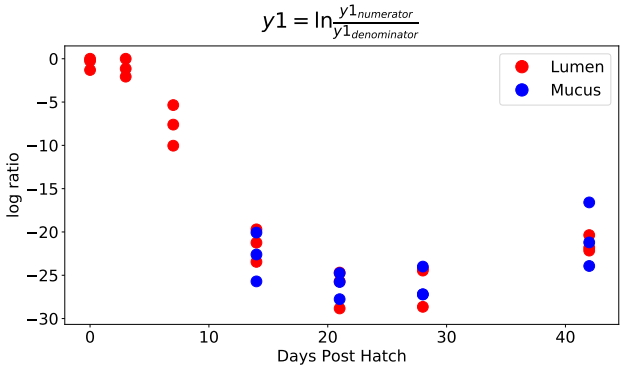


Figure 2.20: ASV log abundance in the caecal microbiome between 0 and 42 d.p.h

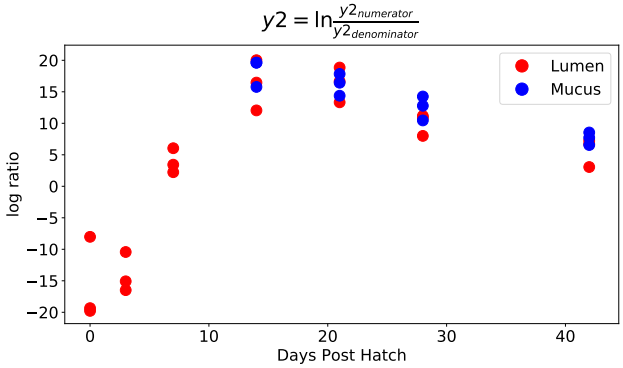
A dendrogram heatmap showing the log abundance of ASVs in caecal lumen and mucus samples grouped by age. Differences in relative abundance between time points are visible in balances y0, y1, y2, y3, y4, y5, y6, y7 and y8 identified by Gneiss analysis as containing differentially abundant ASVs.



(a) Balance y0

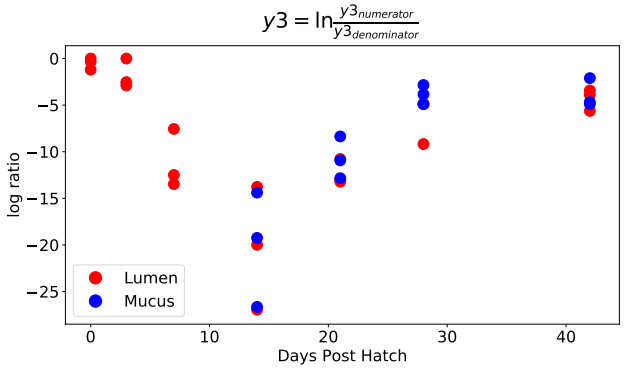


(b) Balance y1

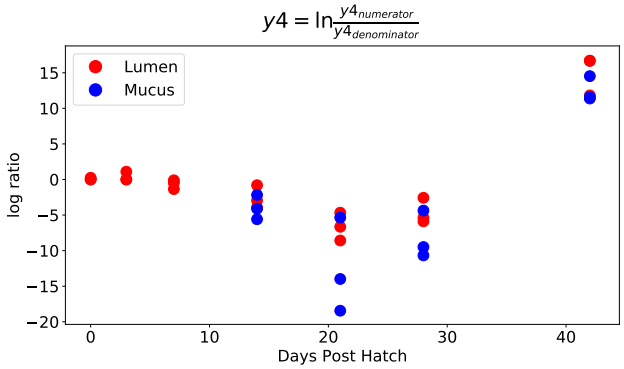


(c) Balance y2

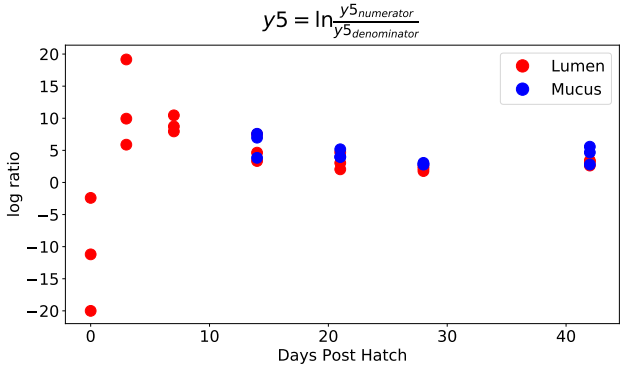
Figure 2.21: Log ratios of balances significantly different between time points during caecal microbiome development



(d) Balance y3

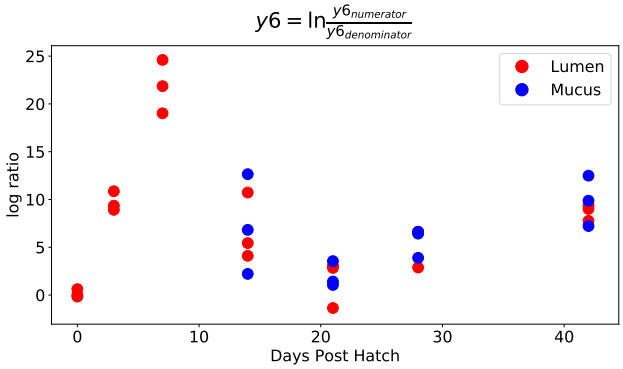


(e) Balance y4

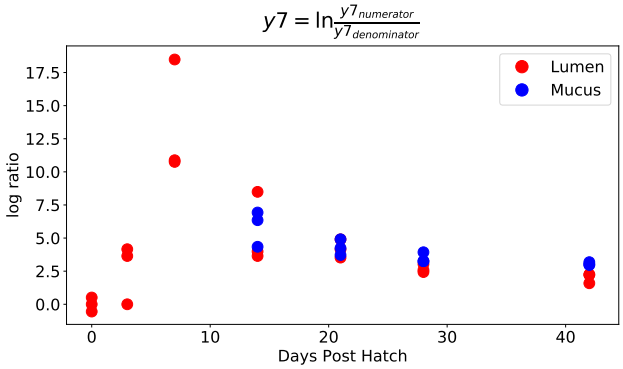


(f) Balance y5

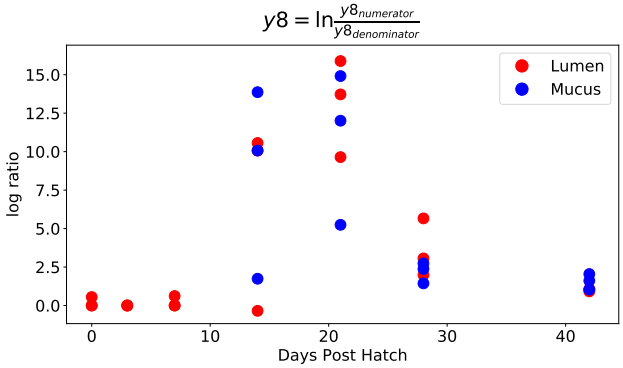
Figure 2.21: Log ratios of balances significantly different between time points during caecal microbiome development



(g) Balance y6



(h) Balance y7



(i) Balance y8

Figure 2.21: Log ratios of balances significantly different between time points during caecal microbiome development

In conjunction with the taxa plot (Figure 2.22) these results demonstrate a pattern of succession within the caecum. Table 2.8 shows the number of ASVs by family identified as differentially abundant at each time point. Most ASVs included in the analysis were assigned to Ruminococcaceae and Lachnospiraceae. Ruminococcaceae was associated with later colonisation of the caecum with most ASVs identified as more abundant from 14 d.p.h. While many Lachnospiraceae ASVs were also identified as more abundant at these later time points there was significant colonisation of the caecum by Lachnospiraceae at 3 and 7 d.p.h. This pattern is visible in the taxaplot since Lachnospiraceae had a higher relative abundance than Ruminococcaceae at 0, 3 and 7 d.p.h. From 14 d.p.h, the relative abundance of these two families was more even. Most ASVs assigned to Ruminococcaceae and Lachnospiraceae were not assigned to a genus, making deeper taxonomic associations with time points difficult to assess. However, some patterns can be discerned at the genus level. Of eight ASVs assigned to *Lachnoclostridium*, a genus of Lachnospiraceae, six were more abundant at 3 d.p.h indicating an ability to colonise the caecum at an early stage whereas ASVs assigned to *Blautia* were more abundant from 14 d.p.h. Some genera of Ruminococcaceae were associated with single time points including *Oscillibacter*, Ruminococcaceae UGC-103 and *Ruminiclostridium* 5 at 14 d.p.h while Ruminococcaceae NK4A214 group and *Faecalibacterium* were more abundant at 28 and 42 d.p.h.

ASVs assigned to Clostridiaceae 1 and Enterobacteriaceae were identified as more abundant at 0 and 3 d.p.h, with the exception of one Clostridiaceae 1 ASV which was more abundant at 21 d.p.h. At the genus level, this ASV was assigned to *Candidatus* Arthromituswhile all other Clostridiaceae 1 ASVs were assigned to *Clostridium sensu stricto* 1 and 2. ASVs assigned to Enterococcaceae were also more abundant at 0 and 3 d.p.h. A search of Enterococcaceae ASVs against the NCBI bacterial 16S rRNA gene database using BLAST revealed a further division with all three Enterococcaceae ASVs abundant at 0 d.p.h closely resembling sequences from *Enterococcus hirae* while all six Enterococcaceae ASVs abundant at 3 d.p.h were most similar to *Enterococcus cecorum*. The association of Clostridiaceae 1, Enterobacteriaceae and Enterococcaceae with an immature microbiome at 0 and 3 d.p.h is supported by the taxa plot where the relative abundance of these three

families decreased substantially from 3 to 7 d.p.h.

Other bacterial families were able to colonise the caecum at 3 d.p.h including Erysipelotrichaceae, Eggerthellaceae, Burkholderiaceae, Peptostreptococcaceae, Coriobacteriaceae, Eubacteriaceae, Bifidobacteriaceae and Lactobacillaceae. Most of these taxa had a low but constant relative abundance within the caecal microbiome from 3 d.p.h onwards, although the relative abundance of Bifidobacteriaceae was high at 3 d.p.h before declining. Lactobacillaceae and Erysipelotrichaceae ASVs were identified as more abundant at later time points. Lactobacillaceae ASVs identified as more abundant at 3 d.p.h most closely resembled *Lactobacillus mucosae*, those at 14 d.p.h were assigned only to *Lactobacillus* and those at 42 d.p.h were assigned to *Lactobacillus vaginalis*. The two Erysipelotrichaceae ASVs identified as more abundant at 3 d.p.h were assigned to *Faecalicoccus* and *Massiliomicrobiota timonensis* whereas those from later time points were either assigned to *Erysipelatoclostridium* or *Turicibacter*. This broadly fits with the succession of Lactobacillaceae and Erysipelotrichaceae ASVs observed in the ileum, although *Erysipelatoclostridium* was identified as an early and transient ileal coloniser whereas in the caecum it was identified as a late coloniser.

Although relatively few ASVs were identified as more abundant at 7 d.p.h, the taxa plot shows that their relative contribution to the caecal microbiome should not be understated. Despite being represented by three ASVs, two of which were more abundant at 7 d.p.h, Bacteroidaceae went on to be one of the most abundant taxa in the caecum.

From 14 d.p.h, a variety of bacterial families were identified as more abundant. These included ASVs assigned to Clostridiales vadin BB60 group, Christensenellaceae, Bacillaceae, Peptococcaceae and a selection of uncultured bacteria belonging to the order Mollicutes RF39. All these families showed a preference for colonising the caecum at later time points. For example, the majority of Christensenellaceae and Bacillaceae ASVs were more abundant at 21 d.p.h while the majority of Clostridiales vadin BB60 group ASVs showed strong colonisation at 28 and 42 d.p.h. The appearance and increases in relative abundance of these taxonomic groups from 14 and 21 d.p.h was confirmed by the taxa plot.

	Number of ASVs						
Family	**Total**	**0 d.p.h**	**3 d.p.h**	**7 d.p.h**	**14 d.p.h**	**21 d.p.h**	**28 & 42 d.p.h** [a]
Ruminococcaceae	163	0	10	4	53	48	48
Lachnospiraceae	121	1	29	9	38	28	16
Clostridiales vadin BB60 group	29	0	0	0	3	9	17
Clostridiaceae 1	19	14	4	0	0	1	0
Enterobacteriaceae	17	12	5	0	0	0	0
Enterococcaceae	9	3	6	0	0	0	0
Christensenellaceae	9	0	0	0	1	6	2
Lactobacillaceae	7	0	3	0	2	0	2
Erysipelotrichaceae	6	0	2	0	0	3	1
Eggerthellaceae	5	0	3	0	0	2	0
Bacillaceae	5	0	0	0	1	4	0
Peptococcaceae	4	0	0	0	0	1	3
Burkholderiaceae	4	0	3	0	1	0	0
Peptostreptococcaceae	4	1	2	0	1	0	0
Bacteroidaceae	3	0	0	2	0	1	0
Coriobacteriaceae	3	0	2	0	0	0	1
Atopobiaceae	2	0	0	1	0	0	1
uncultured rumen bacterium	2	0	0	0	0	2	0
Eubacteriaceae	2	0	2	0	0	0	0
Clostridiales	2	0	0	0	0	0	2
Staphylococcaceae	2	0	0	0	1	1	0
Defluviitaleaceae	1	0	0	0	0	0	1
Bifidobacteriaceae	1	0	1	0	0	0	0
Mollicutes RF39	1	0	0	0	0	0	1
Family XIII	1	0	0	0	0	0	1
Firmicutes bacterium CAG:822	1	0	0	0	0	0	1
gut metagenome	1	0	0	0	0	1	0

[a] Individual taxonomies of significant Gneiss balances are provided in Table A.5.

Table 2.8: Taxonomy of ASVs colonising the caecum at different time points

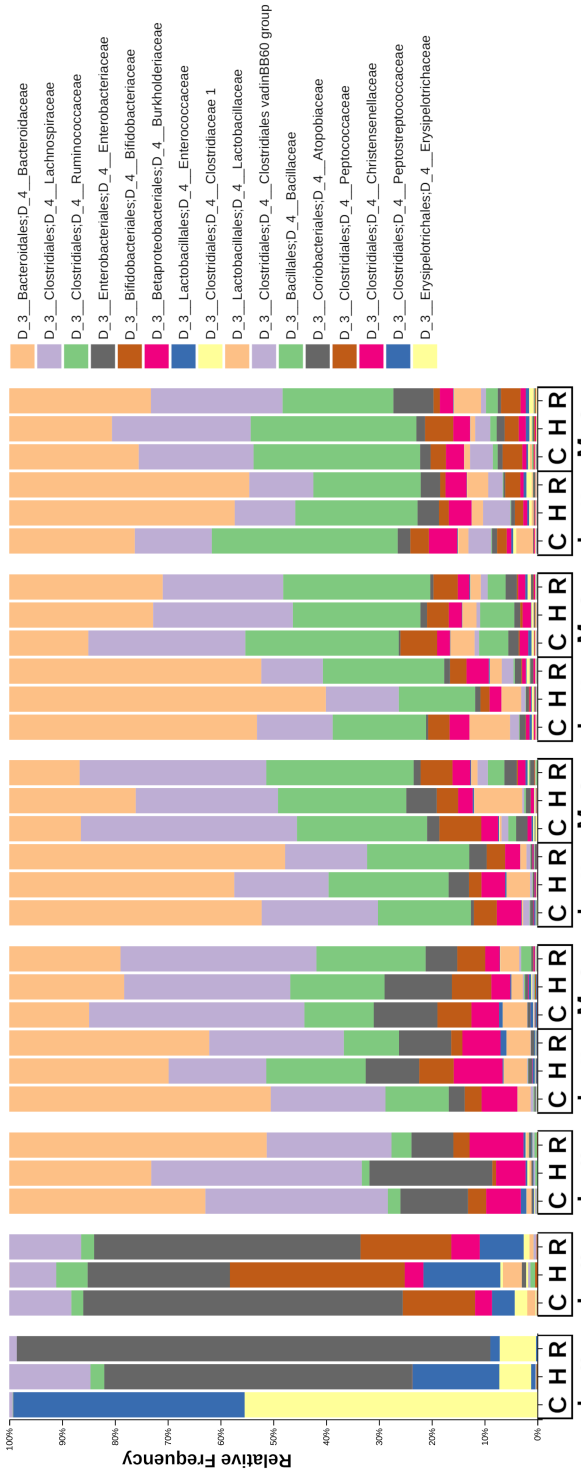


Figure 2.22: Relative abundance of bacterial families in caecal mucus and lumen samples from 0 to 42 d.p.h

Lumen (L); Mucus (M); Cobb (C); Hubbard (H); Ross (R)

2.7.2 Differences between the lumen and mucus-associated microbiomes

Samples from 21, 28 and 42 d.p.h were used to compare lumen and mucus samples from a mature caecal microbiota. Samples from 14 d.p.h were excluded as time post hatch had a small but significant effect on weighted UniFrac distances when samples from 14 d.p.h were included in the analysis (R-statistic = 0.17, p = 0.041).

Alpha diversity and beta diversity

There were no significant differences in alpha diversity between lumen and mucus samples when measured using FPD index. However, mucus samples had a significantly higher SD index than lumen samples (H = 9.83, p = 0.002; Figure 2.18). When measured using an unweighted UniFrac metric, there were no significant differences in beta diversity between lumen and mucus samples (R-statistic = 0.06, p = 0.19) with no clustering of samples by area sampled (Figure 2.19a). However, there was a significant difference in beta diversity when measured using a weighted UniFrac metric (R-statistic = 0.79, p = 0.001). This suggests that while the taxonomic composition of mucus and lumen samples was similar, the distribution of relative abundances was different. Mucus and luminal samples formed distinct clusters on a PCoA plot (Figure 2.19b), although there was one exception to this pattern. The lumen sample which clustered with the mucus samples is from Cobb chickens at 42 d.p.h. With reference to the taxa plots (Figure 2.22), it's clear that the composition of this sample was very similar to mucus taken from Cobb chickens at 42 d.p.h. Results from both alpha and beta diversity suggest that while the taxonomic composition of mucus and lumen samples was similar, there were differences in relative abundance between mucus and lumen samples with a greater evenness in mucus samples.

Differential ASVs between the lumen and mucus microbiomes

Gneiss analysis (Figure 2.24, Table 2.9) and taxa plots (Figure 2.22) revealed differential ASV abundance between mucus and lumen microbiomes. The feature table was filtered to exclude ASVs with a frequency of less than 81 reducing the number of ASVs included from

748 to 375. The overall linear regression model fit was R2 = 0.56 with covariate 'Area' accounting for 7.9% of variance. The log ratio of balance y2 (β = -11.5, $p < 0.001$) was a significant predictor for the covariate of 'Area'.

The log ratio of balance y2 was lower in mucus samples compared to lumen samples showing that the relative abundance of $y2_{denominator}$ ASVs was higher in the mucus than the lumen (Figure 2.23) with this increased abundance visible in the dendrogram heatmap (Figure 2.24). The taxonomy of ASVs identified as more abundant in the mucus or lumen based on balance y2 is presented in Table 2.9.

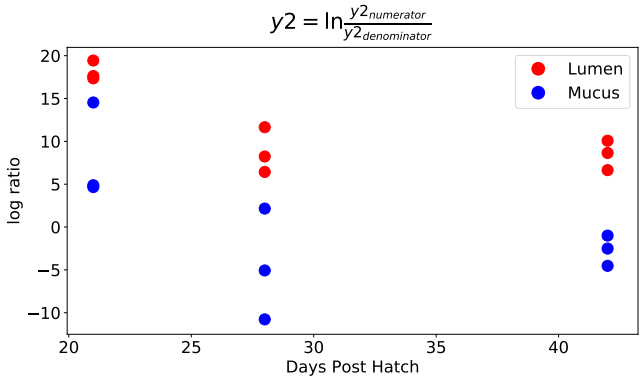


Figure 2.23: Log ratio of balance y2 which was significantly different between caecal mucus and lumen samples

The dendrogram heatmap confirms that the relative abundance of $y2_{denominator}$ ASVs was higher in mucus samples. However, it does not show a pattern of increased relative abundance of $y2_{numerator}$ ASVs in lumen samples compared to mucus samples. As a result, it is difficult to discern which $y2_{numerator}$ ASVs are truly more abundant in the lumen as no other balances are significantly different to allow for greater resolution. However, the taxa plot can aid in this distinction. A paired Student's t-test of relative abundance of Burkholderiaceae (lumen average = 3.97%, mucus average = 2.8%, test statistic = 3.78, p = 0.005) and Bacteroidaceae (lumen average = 45.4%, mucus average = 21.4%, test statistic = 5.97, $p < 0.001$) showed significant differences in relative abundance between lumen and mucus samples. On the other hand, some taxa in $y2_{numerator}$ showed a significantly higher relative abundance in mucus including Bifidobacteriaceae (lumen average = 2.8%,

mucus average = 4.8%, test statistic = 4.11, p = 0.003), Atopobiaceae (lumen average = 0.68%, mucus average = 1.53%, test statistic = 4.04, p = 0.003) and Eubacteriaceae (lumen average = 0.02%, mucus average = 0.06%, test statistic = 3.11, p = 0.01). It's possible that since these taxa are represented by one or two ASVs that the limited sample size did not provide sufficient statistical power to demonstrate a significant difference in their abundance between mucus and lumen samples using Gneiss analysis. As a result, there is some doubt as to whether these taxa are differentially abundant between the mucus and the lumen.

Differences between the lumen and mucus microbiomes confirmed by quantitative PCR

Based on the results from Gneiss analysis and the taxa plot, quantitative PCR assays for Bacteroidaceae, Lachnospiraceae, Ruminococcaceae, Bifidobacteriaceae and Bacillaceae were performed. A combined primer pair for Lachnospiraceae and Ruminococcaceae was used as well as specific primers for Clostridium cluster XIVa&b and Clostridium cluster IV with the former corresponding roughly to Lachnospiraceae and the latter to Ruminococcaceae. Previously published primers were used for Bacteroides and Bifidobacterium detection as well-trialled primer pairs were available in the literature. The primers for the Bacillaceae ASVs were generated as described in Section 2.2.7.

Results showed the same pattern as detected by Gneiss analysis (Figure 2.25). Bacteroidaceae were significantly more abundant in lumen samples (test statistic = 6.3, $p < 0.001$) and Bacillaceae (test statistic = -2.4, p = 0.03) were significantly more abundant in the mucus. Bifidobacteriaceae were significantly more abundant in the mucus (test statistic = -2.5, p = 0.03) although this pattern was not present at 21 d.p.h. There was a significantly higher abundance of Lachnospiraceae-Ruminococcaceae, Clostridium cluster IV and Clostridium cluster XIVa&b in the mucus compared to the lumen (test statistic = -6.7, $p < 0.001$, test statistic = -9.1, $p < 0.001$ and test statistic = -5.8, $p < 0.001$ respectively).

The correlation between abundance determined by qPCR and relative abundance determined by sequencing varied between primers. There was a strong, positive correlation

		Number of ASVs		
Taxonomy	**Total**	**Lumen**	**Mucus**	**NDA** [a]
Ruminococcaceae	163	83	25	55
Lachnospiraceae	114	75	20	19
Clostridiales vadin BB60 group	31	9	0	22
Christensenellaceae	10	1	5	4
Lactobacillaceae	6	4	0	2
Bacillaceae	5	0	4	1
Burkholderiaceae	4	4	0	0
Erysipelotrichaceae	4	1	1	2
Clostridiales	4	0	0	4
Peptococcaceae	4	0	1	3
Eggerthellaceae	3	1	2	0
Bacteroidaceae	3	3	0	0
uncultured rumen bacterium	2	1	0	1
Coriobacteriaceae	2	0	1	1
Atopobiaceae	2	1	0	1
Peptostreptococcaceae	2	1	1	0
Enterococcaceae	2	1	1	0
Enterobacteriaceae	2	2	0	0
Family XIII	2	0	0	2
Mollicutes RF39	2	1	0	1
Defluviitaleaceae	2	1	0	1
gut metagenome	1	0	0	1
Clostridiaceae 1	1	1	0	0
Firmicutes bacterium CAG:822	1	0	1	0
Staphylococcaceae	1	1	0	0
Eubacteriaceae	1	1	0	0

[a] ASVs defined as NDA were not differentially abundant between mucus and lumen samples. Individual taxonomies of significant Gneiss balances are provided in Figure A.6.

Table 2.9: Taxonomy of differentially abundant ASVs between caecal mucus and lumen samples

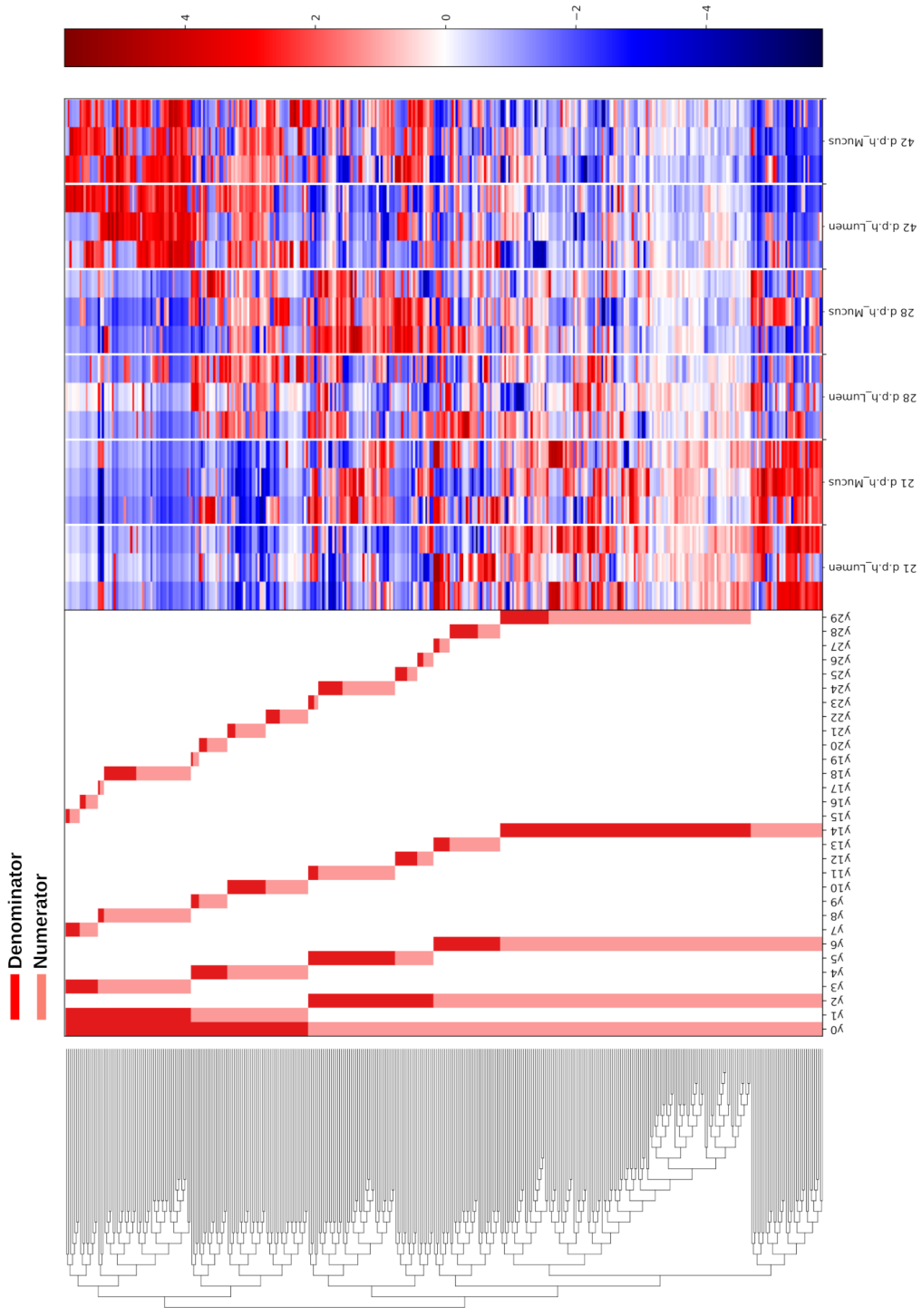


Figure 2.24: Dendrogram heatmap of ASV abundance showing differences in caecal mucus and lumen microbiome composition between 21 and 42 d.p.h

and Lachnospiraceae-Ruminococcaceae (rs(22) = 0.40, p = 0.05).

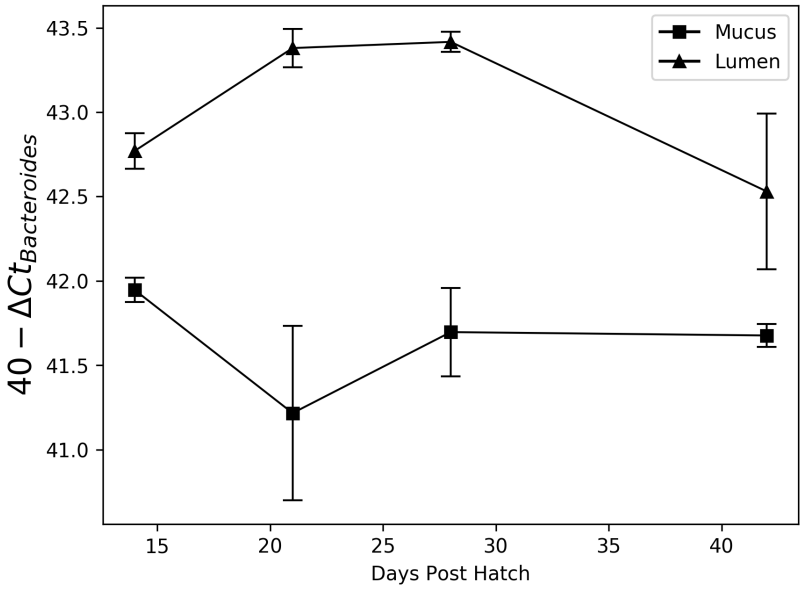
2.7.3 Breed differences in the caecal microbiome

Individual samples taken from caecal mucus at 42 d.p.h were sequenced to investigate breed differences in microbiome composition. The taxa plots of pooled samples showed some early differences in the caecal microbiota between breeds (Figure 2.22). At 0 d.p.h, there were large differences in composition between the breeds with Hubbard and Ross birds mainly colonised by Enterobacteriaceae while Cobb birds were colonised by Enterococcaceae and Clostridiaceae. By 3 d.p.h, there was increased homogeneity between the breeds although Hubbard birds had proportionally more Bifidobacteriaceae and less Enterobacteriaceae than the other two breeds. From 7 d.p.h, there were no visible differences between breeds in the taxa plots.

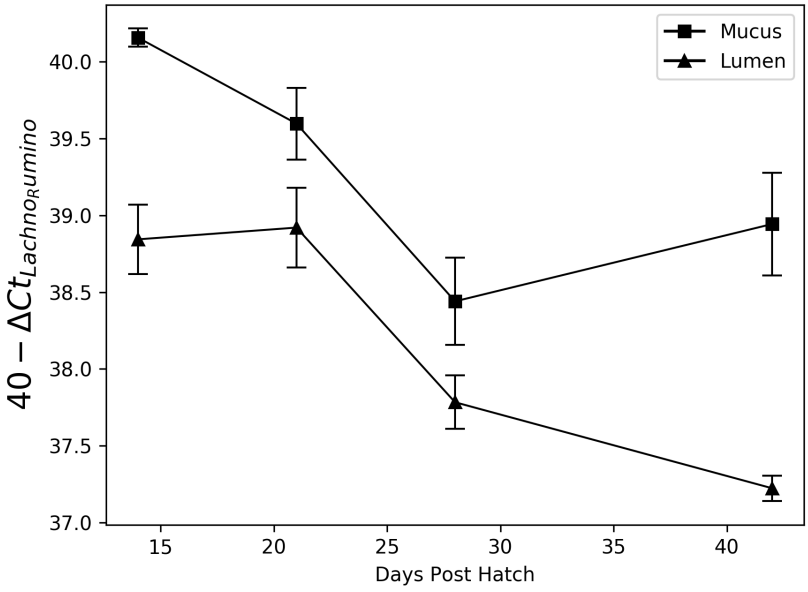
Alpha and beta diversity

Using data from 5 samples of caecal mucus from each breed taken at 42 d.p.h some differences in alpha and beta diversity were noted. Cobb broilers had a significantly higher FPD index than Ross broilers (H = 5.77, p = 0.05; Figure 2.26). Hubbard broilers also had a higher FPD index than Ross broilers, although the difference was not significant (H = 3.93, p = 0.07). There was no significant difference in FPD index between Cobb and Hubbard broilers (H = 0.27, p = 0.6). Similarly, Cobb broilers had a higher average SD index than Ross broilers, although this difference was not significant (H = 3.15, p = 0.11). Hubbard broilers also had a significantly higher average SD index than Ross broilers (H = 5.77, p = 0.05) but there was no significant difference in SD index between Cobb and Hubbard broilers (H = 0.88, p = 0.35).

Breed had a significant effect on beta diversity (R-statistic = 0.25, p = 0.03). Pair-wise tests between breeds revealed that there was no significant difference in weighted UniFrac distance between samples from Cobb and Hubbard birds and samples taken from Ross and Hubbard birds. There was a significant difference between samples taken from Cobb and Ross birds (ANOSIM: R-statistic = 0.57, p = 0.009).

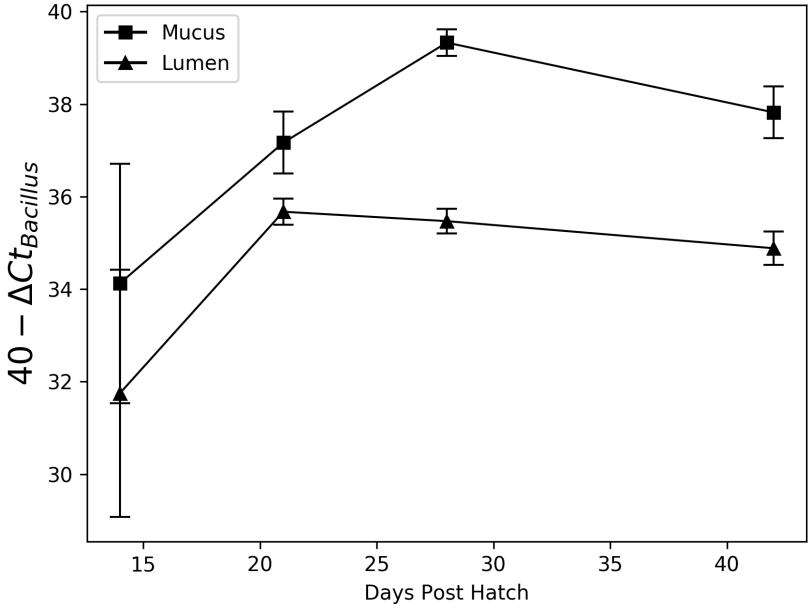


(a) Bacteroides

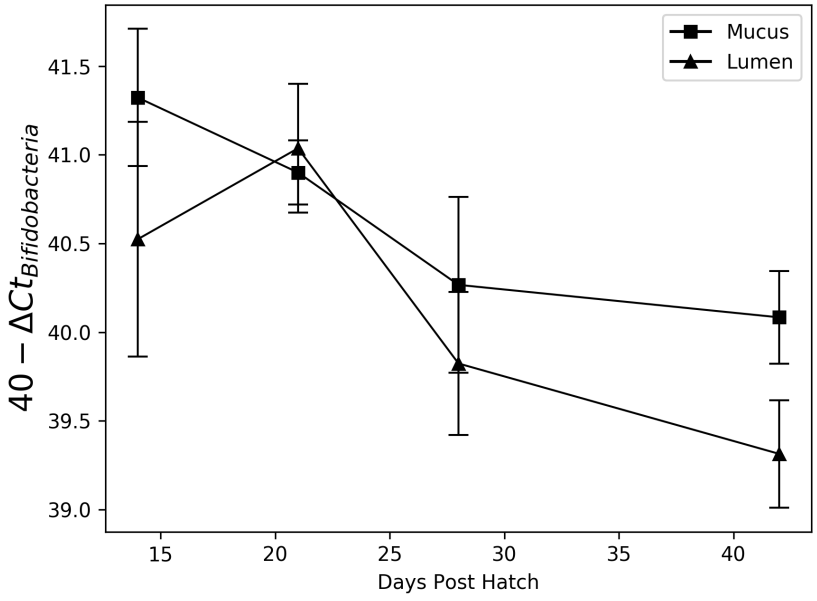


(b) Lachnospiraceae and Ruminococcaceae

Figure 2.25: Absolute abundance of taxa in the caecal lumen and mucus determined by qPCR

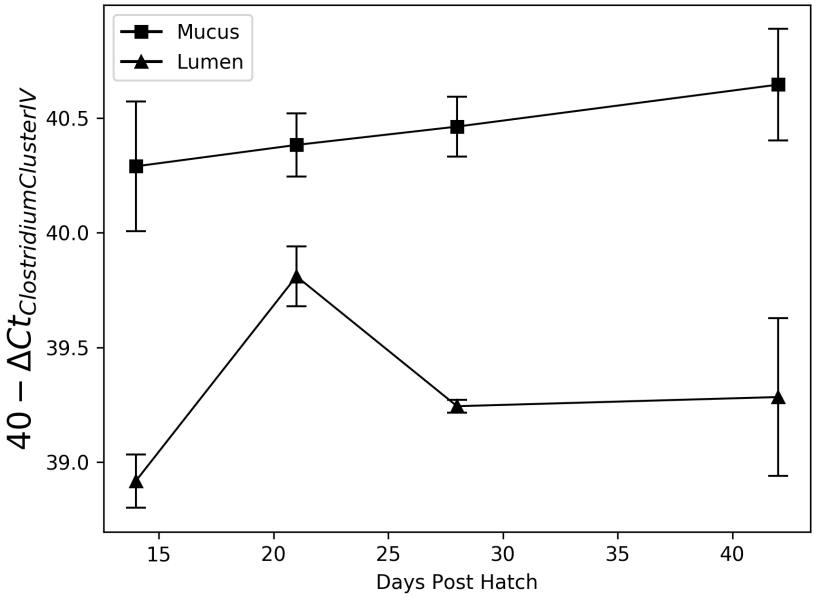


(c) Bacillus

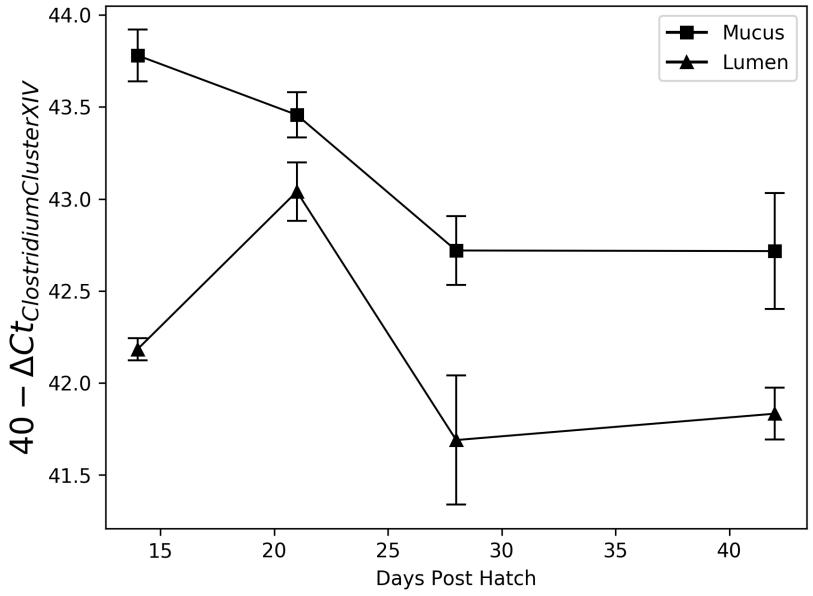


(d) Bifidobacteria

Figure 2.25: Absolute abundance of taxa in the caecal lumen and mucus determined by qPCR

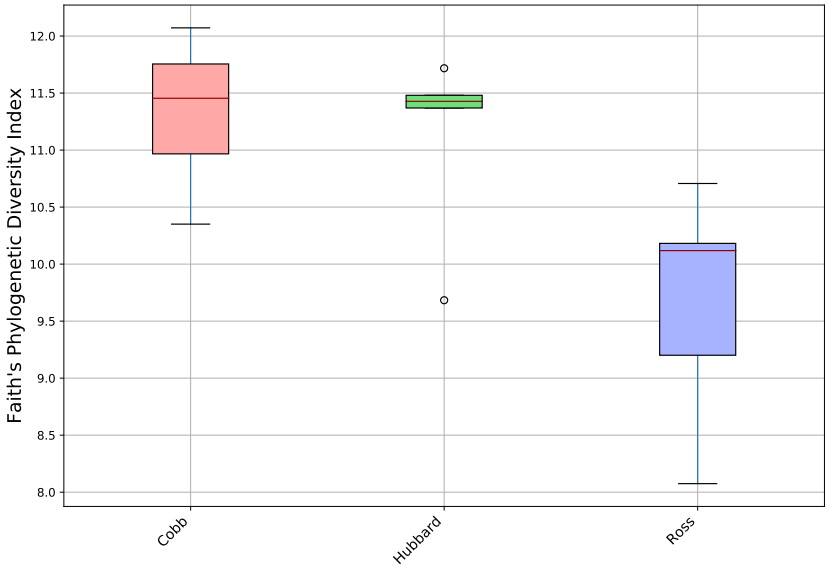


(e) Clostridium Cluster IV

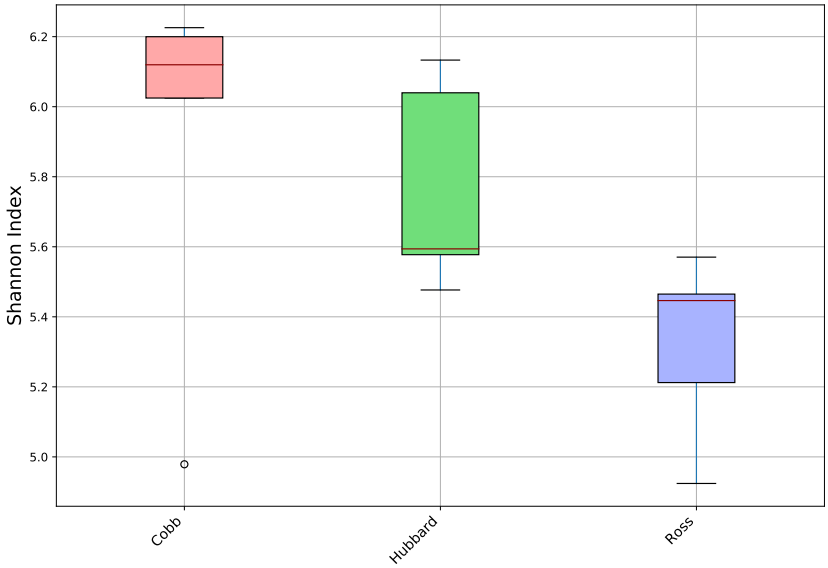


(f) Clostridium Cluster XIV

Figure 2.25: Absolute abundance of taxa in the caecal lumen and mucus determined by qPCR



(a) FPD Index



(b) SD Index

Figure 2.26: Alpha diversity in caecal mucus samples taken from Hubbard, Ross and Cobb chickens at 42 d.p.h

Differential ASVs between breeds

The feature table was filtered to exclude ASVs with a frequency of less than 66, reducing the number of ASVs included from 790 to 398. Pairwise Gneiss analysis between breeds revealed differential ASV abundance between caecal mucus microbiota in Cobb and Ross chickens (Table 2.10) with fewer differences discovered between Hubbard and Ross chickens and between Hubbard and Cobb chickens.

For the comparison between Cobb and Ross chickens, the covariate 'Breed' accounted for 15.3% of variance. The log ratio of balances y2 (β = -13.7, p = 0.01) and y14 (β = 12.2, p = 0.001) were significant predictors for the covariate of 'Breed'. The log ratio of balance y2 was, on average, lower in Ross chickens showing a higher relative abundance of denominator ASVs. This difference in abundance is visible on the dendrogram heatmap (Figure A.1). The log ratio of balance y14 was lower in Cobb chickens. The dendrogram heatmap shows that this was due to an increased relative abundance of $y14_{denominator}$ ASVs in samples taken from Cobb chickens. The taxonomy of ASVs in these balances is displayed in Table 2.10.

For the comparison between Hubbard and Ross chickens, the covariate of 'Breed' accounted for 12.4% of variance. Log ratio of balance y1 was approaching significance (β = 8.22, p = 0.07) and on review of the dendrogram heatmap (Figure A.2) was included in the analysis. The log ratio of balance y1 was lower in samples from Hubbard chickens. This difference was due to an increased relative abundance of $y1_{denominator}$ ASVs which can be seen in the dendrogram heatmap. These ASVs were mainly assigned to Ruminococcaceae (n = 21), Lachnospiraceae (n = 7) and Clostridiales vadin BB60 group (n = 6). The remaining ASVs were assigned to Christensenellaceae (n = 2) and one each to Peptococcaceae, Enterococcaceae, Clostridiales Family XIII and Eggerthellaceae.

For the comparison between Hubbard and Cobb chickens, the covariate of 'Breed' accounted for 9.7% of variance. Log ratio of balance y15 (β = 4.13, p = 0.01) was a significant predictor for the covariate of 'Breed'. The log ratio of balance y15 was lower in Cobb chickens suggesting that $y15_{denominator}$ ASVs were more abundant. This is confirmed

by the dendrogram heatmap (Figure A.3). The ASVs composing $y15_{denominator}$ were assigned to Ruminococcaceae (n = 3) and Coriobacteriaceae (n = 1).

		Number of ASVs		
Taxonomy	**Total**	**Ross**	**Cobb**	**NDA** [a]
Ruminococcaceae	172	21	39	112
Lachnospiraceae	113	15	17	81
Clostridiales vadin BB60 group	35	6	14	15
Christensenellaceae	11	1	4	6
Peptococcaceae	8	0	2	6
Lactobacillaceae	7	6	0	1
Bacillaceae	5	1	1	3
Family XIII	5	1	2	2
Burkholderiaceae	4	0	0	4
Clostridiales	4	1	1	2
Erysipelotrichaceae	4	1	1	2
Eggerthellaceae	3	1	1	1
Coriobacteriaceae	2	0	1	1
Bacteroidaceae	2	0	0	2
Enterobacteriaceae	2	0	0	2
uncultured rumen bacterium	2	0	1	1
Firmicutes bacterium CAG:822	2	0	2	0
Defluviitaleaceae	2	1	1	0
Enterococcaceae	2	1	0	1
Atopobiaceae	2	0	0	2
Peptostreptococcaceae	2	0	0	2
uncultured bacterium	1	0	0	1
Clostridiaceae 1	1	0	1	0
gut metagenome	1	0	0	1
Mollicutes RF39	1	0	1	0

[a] ASVs defined as NDA were not differentially abundant between samples taken from Ross and Cobb chickens. Individual taxonomies of significant Gneiss balances are provided in Figure A.1.

Table 2.10: Taxonomy of differentially abundant ASVs between caecal mucus samples from Ross and Cobb chickens at 42 d.p.h.

2.7.4 Discussion

The analysis presented above aimed to characterise the succession of bacteria in the caecal microbiota in three broiler breeds between 0 and 42 d.p.h. The results of this study must be

interpreted in light of small sample sizes and the pooling of samples which limit statistical power and obscures the inherent variability of individual microbiota composition respectively. However, broad patterns are visible within the data and merit further discussion.

Bacterial colonisation of the caecum

The initial microbiome observed in caeca from Cobb, Ross and Hubbard chickens was very similar to that described in other studies (Ballou *et al.*, 2016). Large differences in microbiome composition were observed between breeds immediately post hatch. This is most likely due to different bacterial exposure between handling of the chicks from hatch to collection (Pedroso *et al.*, 2005). These early differences in microbiota composition did not affect the subsequent development of a mature microbiota. Differences between breeds were still visible at 3 and 7 d.p.h but were no longer visible at 14 d.p.h. This suggests that environmental exposure, diet and management practices were more important than genetics in shaping the caecal microbiota.

The rise of Bifidobacteriaceae at 3.d.p.h may represent an important step in the maturation of the caecal microbiota as a stimulus for Bacteroidaceae growth. Recent studies have shown that *Bacteroides fragilis* and other intestinal bacteria can metabolise exopolysaccharides, a complex carbohydrate produced by some *Bifidobacterium* strains (Rios-Covian *et al.*, 2013; Salazar *et al.*, 2008). It's possible that an initial rise in *Bifidobacterium* produces polysaccharides which are then used by other bacteria as a substrate and the basis for the expansion of their populations. Salazar *et al.* (2008) also showed that exopolysaccharides from strains of *Bifidobacterium* supported populations of bacteria such as *Faecalibacterium prausnitzii*. This suggests that the initial colonising strains of Bifidobacteriaceae could influence future microbiota development in the caecum.

As well as promoting the growth of other caecal microbes Bifidobacteriaceae play an important role in pathogen exclusion and intestinal barrier function (Sanz *et al.*, 2016; Ling *et al.*, 2016). Most of the evidence for this comes from mammalian studies, however, a *Bifidobacterium* probiotic has been shown to improve epithelial integrity in chickens (Song *et al.*, 2014). The mechanism may be associated with *Bifidobacterium*'s production of

acetate which can protect mice epithelial cells in the face of *Escherichia coli* O157 infection (Fukuda *et al.*, 2012). *Bifidobacterium* also plays a role in dendritic cell maturation and the balancing of regulatory T and T helper 17 cell development (López *et al.*, 2011; Dong *et al.*, 2010), similar to the role of *Candidatus* Arthromitus in the ileum.

One of the caecum's main functions is bacterial fermentation of indigestible polysaccharides to produce short chain fatty acids (SCFAs) which can be utilised by the host's epithelial cells. The taxa which fulfil this role are certain classes of Firmicutes (such as Lachnospiraceae or Ruminococcaceae) and Bacteroidetes. In this study, these two classes were equally abundant with some Firmicutes localising to the mucus and Bacteroidetes to the lumen. Previous studies have found that either Firmicutes or Bacteroidetes is more abundant in the caecum and is often discussed in terms of the Firmicutes:Bacteroidetes (F/B) ratio (Oakley *et al.*, 2014*a*; Wise and Siragusa, 2007; Zhu and Joerger, 2003). This discrepancy between studies may be explained by differences in methodology or sampling. However, it has been reported that even among chickens from the same flock, the F/B ratio can vary substantially (Stanley *et al.*, 2013*b*). There is conflicting evidence as to the F/B ratio's impact on metabolism and feed conversion ratio (FCR). Stanley *et al.* (2013*b*) reported that the differences found between chickens in the same flock didn't appear to have an effect on apparent metabolisable energy or FCR. In contrast to this result, a comparison of the faecal microbiota between high and low FCR broiler chickens at 49 d.p.h found significant differences in microbiota composition between the two groups. Low FCR birds had a higher F/B ratio than high FCR birds suggesting that a higher relative abundance of Firmicutes can be associated with increased metabolic efficiency (Singh *et al.*, 2012). Similar patterns have been noted in the human microbiota with a higher F/B ratio linked to obesity (Ley *et al.*, 2006). However, Firmicutes is a diverse phylum and closer inspection at a lower taxonomic level reveals a more complex pattern. The two major families of caecal Firmicutes have been identified as Ruminococcaceae and Lachnospiraceae. Singh *et al.* (2012) found that the relative abundance of Lachnospiraceae was nearly twice as high in high FCR birds while the relative abundance of Ruminococcaceae was 15 times higher in low FCR birds.

Lachnospiraceae were the first Clostridia to colonise the caecum in this study. In contrast, Ruminococcaceae were relatively late colonisers which appeared to replace Lachnospiraceae, especially in the lumen, at later time points. These two families are poorly classified, and material related to their potential role in the microbiome is scarce. Differences in gene abundance between genomes of Lachnospiraceae and Ruminococcaceae have been reported (Biddle *et al.*, 2013). However, the functional significance of these differences in relation to the pattern of succession is not clear. One exception to this rule is *Faecalibacterium prausnitzii*, a butyrate producing member of Ruminococcaceae (Bjerrum *et al.*, 2006; Eeckhaut *et al.*, 2011), which has been identified as a potentially beneficial microbe (Torok *et al.*, 2011). Aside from its prominence as a late stage member of the caecal microbiota little is known about the impact of *F. prausnitzii* on the chicken. Some inferences can be made from observations reported in mouse and human studies. In these species, *F. prausnitzii* is thought to have an anti-inflammatory effect since lower counts are observed in various inflammatory diseases (Sokol *et al.*, 2008, 2009). Additionally, inflammation and intestinal barrier function are improved in a mouse inflammatory bowel disease model by the addition of *F. prausnitzii* (Carlsson *et al.*, 2013; Sokol *et al.*, 2008). While the production of butyrate may play a role in *F. prausnitzii*'s beneficial effects, the possibility of a more complex mechanism involving the production of other molecules should not be ruled out (Van Immerseel *et al.*, 2010). The results of this study also showed later colonisation of the caecum by other members of Firmicutes such as Christensenellaceae, Clostridiales vadin BB60 group, Peptococcaceae and Bacillaceae, as well as Mollicutes RF39. Many of these families are poorly classified and little is known about their metabolism or role in the microbiota. One exception is the family Christensenellaceae which has been linked to lower body mass index and improved overall gut health in humans (Garcia-Mantrana *et al.*, 2018; Goodrich *et al.*, 2014).

Another feature of the developing microbiota in this study was the decrease in Enterobacteriaceae over time. This bacterial family is considered of importance in poultry production, not just as pathogens, but also due to their carriage of genetic factors responsible for antimicrobial resistance (Saliu *et al.*, 2017). The relative abundance of Enterobacteriaceae

was highest at 0 and 3 d.p.h before decreasing to its lowest relative abundance at 21 d.p.h. A similar pattern of replacement of Enterobacteriaceae by other taxa over the first few weeks of life has been observed in previous studies (Ballou *et al.*, 2016; Schokker *et al.*, 2015; Videnska *et al.*, 2014). Reduced abundance and growth of Enterobacteriaceae have been linked to levels of SCFAs like acetate, butyrate and propionate both *in vitro* and *in vivo* (Van Der Wielen *et al.*, 2000). As such, the reduction in Enterobacteriaceae observed in this study and others could be attributed to caecal colonisation by Lachnospiraceae, Ruminococcaceae and Bacteroidaceae. This highlights the importance of these taxa and suggests that interventions which promote their early colonisation should be prioritised.

The order of caecal bacterial succession described above may not be representative of a commercial setting where environmental exposure and other factors will allow for a differing microbiome to develop. However, it does raise questions about the general mechanisms of succession. It's possible that the late arrival of taxa such as Clostridiales vadin BB60 group and Bacillaceae was a question of environmental exposure, that these taxonomic groups were not present in the environment until 14 or 21 d.p.h. However, it also raises the possibility that these taxa have some prerequisite conditions that must be fulfilled, either by the host or earlier bacterial colonisers, before they can establish a population in the caecum. Identifying factors or interventions that accelerate the development of a mature microbiota may provide further benefits to chicken production in terms of increased yields or reduced losses to infectious disease.

The mucus and lumen-associated microbiomes are different

In this study, a higher relative abundance of Bacteroidaceae was found in the lumen while Lachnospiraceae, Ruminococcaceae, Bifidobacteriaceae and Bacillaceae were more abundant in the mucus. Similar differences between mucus and lumen microbiota have been described in other species. Higher levels of Lachnospiraceae and a lower abundance of Bacteroidaceae have been reported in mouse colon mucus when compared to the lumen (Nava *et al.*, 2011). This may be due to the differing energy sources of these bacteria. As in other animals, bacterial degradation of indigestible polysaccharides into SCFAs plays an important role in

host nutrition. In the human gut, *Bacteroides* have the highest number and diversity of genes associated with polysaccharide metabolism (Kaoutari *et al.*, 2013). This is also true of chickens with metagenomic studies finding polysaccharide utilisation systems associated with caecal Bacteroidetes (Sergeant *et al.*, 2014). It would make sense for Bacteroidaceae abundance to be higher in the lumen where their energy source is most abundant. The same reasoning can explain the higher abundance of Lachnospiraceae in mucus. A recent metagenomic analysis of Lachnospiraceae and Ruminococcaceae genomes showed differences in gene abundance related to carbohydrate metabolism. Ruminococcaceae had higher numbers of cellulase and xylanase genes related to fermentation of substrates which are more abundant in the lumen. Lachnospiraceae were found to have a higher number of genes for glycoside hydrolase family 13, a group of enzymes associated with cleavage of α–amylase bonds present in starch and glycogen (Biddle *et al.*, 2013).

Members of the Lachnospiraceae family have also been shown to utilise mucin glycans as a sole carbon source, producing propanol and propionate (Crost *et al.*, 2013). Due to the preference for some Lachnospiraceae ASVs to reside in the mucus it is likely that these strains also possess enzymes which allow them to utilise host mucins as an energy source. Equally, strains of Coriobacteriaceae and Bifidobacteriaceae have been found with the ability to degrade mucin in other species (Looft *et al.*, 2015; Tailford *et al.*, 2015). The ability of Bifidobacteriaceae to adhere to mucus is well documented and also explains higher abundance in the mucus than the lumen (Collado *et al.*, 2005; He *et al.*, 2001; Ouwehand *et al.*, 2000). The host likely benefits from abundant Firmicutes in mucus. Differences in SCFA production have been observed between Firmicutes and Bacteroides in the chicken caecum with the former producing butyrate while the latter produces mainly propionate (Polansky *et al.*, 2016). Since, epithelial cells utilise butyrate as their principal energy source, a ready supply in the mucus is likely to support early epithelial maturation and growth (Polansky *et al.*, 2016).

While these results can be explained by bacterial adaptation to niches based on substrate availability, an alternative explanation may lie in host regulation of the microbiota and maturation of the immune system. In this study, there was a pattern of mucosal colonisation

by ASVs which appeared in the caecum before 14 d.p.h. While the association between appearance in the microbiota and the ability to colonise caecal mucus may be incidental since Lachnospiraceae colonised the caecum before Ruminococcaceae, it may be that early presence in the caecum results in host tolerance of certain ASVs allowing them to occupy the mucus layer.

These results have implications for other studies of the chicken caecal microbiome. For example, studies observing the effects of probiotics containing Bifidobacteriaceae or Bacillaceae which observe only the lumen microbiota may not detect increased abundance of these taxa. Additionally, the F/B ratio is often used to compare microbiota between groups (Ley *et al.*, 2006; Li *et al.*, 2017; Wang *et al.*, 2017). One notable study using this parameter found a large range in F/B ratio among individuals of the same breed housed under the same conditions (Stanley *et al.*, 2013*a*). It is possible that some of this variation could be due to different ratios of lumen content to mucus in samples. This would be particularly noticeable in chickens which had empty caeca at the time of sampling since any content present is likely to have a high proportion of mucus. Many studies of the caecal microbiome do not detail the sampling methodology enough to discern whether samples were taken from the lumen or the mucus. These results further emphasise the importance of a detailed methodology which differentiates between sampling from different compartments of the caecum.

Differences between breeds

Some differences between breeds were visible in the pooled samples. These differences occurred at 0 and 3 d.p.h with the greatest present at 0 d.p.h. At subsequent time points there were no significant differences between pooled samples from different breeds. The difference in early microbiota can be explained by the previous finding that chicks from different hatcheries are colonised by different microbes (Pedroso *et al.*, 2005). Although the chicks used in this experiment were sourced from the same hatchery, chicks from different breeds are hatched in different buildings resulting in different environmental exposure. As such, the differences between breeds at 0 d.p.h cannot be attributed to genotype since it is

likely that chicks are colonised by whichever environmental bacteria are present at the time of hatch and this will vary between hatcheries and within hatcheries.

Analysis of five samples of caecal mucus taken from each breed at 42 d.p.h showed differences in microbiota between breeds. The results of alpha and beta diversity, as well as Gneiss analysis, suggest that Cobb and Hubbard broilers had the most similar microbiota composition with the greatest differences found between Cobb and Ross broilers. Many of the ASVs that were differentially abundant between Cobb and Ross broilers were assigned to Ruminococcaceae and Lachnospiraceae, perhaps because these were among the most prevalent taxa in the caecum. It is of interest that most Lactobacillaceae were more abundant in samples from Ross broilers while a high proportion of Christensenellaceae were more abundant in Cobb broilers. Both of these taxa have been linked to metabolic effects on the host. Dietary supplementation with *Lactobacillus* strains has been previously demonstrated to improve body weight gain and FCR in chickens (Kalavathy *et al.*, 2003) while Christensenellaceae is more abundant in the microbiota of lean compared to obese humans (Garcia-Mantrana *et al.*, 2018; Goodrich *et al.*, 2014). However, in the absence of detailed performance data such as FCR or body composition analysis, it is not possible to ascribe a functional or practical significance to these results. Greater taxonomic resolution or metagenomic analysis would also be required as the metabolic effects of bacteria is likely to be strain dependent.

Another factor which may have affected these results is the relative composition of lumen contents to mucus of the samples. It was noted earlier that the pooled lumen sample from Cobb broilers at 42 d.p.h was the only lumen sample with a similar beta diversity to mucus samples. Since it has already been demonstrated that there are significant differences between lumen and mucus microbiota, the observed difference could be attributed to a random sampling error amplified by the small sample sizes used in this study. It is possible that samples taken from Ross broilers had more content or less mucus compared to those from Cobb broilers, accounting for the differences between them. Caecal emptying occurs several times a day in chickens. A recently emptied caecum would have a higher proportion of mucus to luminal contents during sampling which may yield different results had the

caecum been full. With this in mind, while this experiment showed some differences in microbiota composition between genotypes, clear results are hampered by small sample size. However, an influence of genotype on caecal microbiota cannot be discounted.

2.7.5 Summary

This study observed the development of the caecal microbiota between hatch and 42 d.p.h, covering the lifespan of a modern broiler chicken. After hatch, the microbiota had poor diversity and was mainly composed of environmental bacteria. Between hatch and 21 d.p.h, the microbiome became more complex and matured to a diverse community. Significant differences were found in the composition of lumen and mucus microbiomes with Bacteroidaceae more abundant in the lumen and some Lachnospiraceae, Ruminococcaceae, Bacillaceae and Bifidobacteriaceae more abundant in the mucus. Although some significant differences were found in the microbiota between breeds, it is possible that these differences were caused by other factors such as a higher relative composition of caecal content to mucus.

2.8 Conclusion

This chapter presented an experiment designed to observe the normal development of the microbiome in the ileum and caecum between 0 and 42 d.p.h. This study confirmed the findings of previous work which suggests that the caecal and ileal microbiomes immediately post-hatch is composed of a few taxa of environmental bacteria. These are quickly replaced by 3 and 7 d.p.h by a more typical bacterial community. The ileal and caecal microbiomes began to diverge from 3 d.p.h. In the ileum, key landmarks in succession were identified including the first colonisation by *Lactobacillus* at 3 d.p.h, increased relative abundance of *Candidatus* Arthromitus between 7 and 14 d.p.h and colonisation by slower growing genera such as *Romboutsia* and *Turicibacter* between 14 and 21 d.p.h. Although a high abundance of *Lactobacillus* was observed this was a transient feature despite *Lactobacillus*' status as a hallmark of the small intestinal microbiome. In the caecum, microbiome diversity

increased from 0 to 42 d.p.h with new taxa continuing to join the community until 42 d.p.h although the community evenness did not increase from 14 d.p.h. A stable community was present from between 14 and 21 d.p.h. It is worth noting that for more than half of the production period the ileal and caecal micorbiomes were developing and would be more susceptible to changes brought about by external factors. Earlier interventions in the microbiota are likely to be more successful, both in terms of altering the mature microbiota and optimizing the microbiota's impact on metabolic function and immune system maturation. Interventions should focus on promoting early maturation particularly with respect to Bacteroidaceae, Lachnospiraceae and Ruminococcaceae in the caecum and *Candidatus* Arthromitus, *Romboutsia* and *Turicibacter* in the ileum.

Significant differences between lumen and mucus microbiomes were found in both the caecum and the ileum. In the ileum, the relative abundance of *Candidatus* Arthromitus was higher in the mucus along with several taxa more associated with the caecal microbiome. Equally, in the caecal microbiome there was a higher abundance of Bacteroidaceae in the lumen while Clostridiales such as Lachnospiraceae and Ruminococcaceae were more abundant in the mucus along with Bifidobacteriaceae and Bacillaceae. These differences in lumen and mucus microbiomes highlight the importance for accurate methodologies which specify whether samples were representative of both niches within an organ.

Some breed differences were identified during the study. In the ileum, the pattern of succession described above was similar between all three breeds. However, the ileal microbiome of Ross broilers developed quicker than that of Hubbard or Cobb birds. This was especially evident in the increase of *Candidatus* Arthromitus abundance, which could explain differences in the susceptibility to infectious enteric disease previously observed between these breeds. In the caecum, there were some minor differences in microbiome composition between breeds however, it's possible that differences in caecal content to mucus composition of the samples could have contributed to the differences found with small sample sizes hampering efforts to distinguish between sample variation and true biological variation.

Chapter 3

Altering the Early Microbiome Using Topical Egg Treatments

3.1 Introduction

As previously discussed, the intestinal microbiome plays an important role in host health and metabolism. The role of the microbiome is especially crucial in early life for development of the immune system. Normally, neonatal animals are colonised by bacteria from the mother and environment. Industrialisation of poultry production has led to separation of mature adults, eggs and immature chickens at independent sites. Breeder flocks provide fertile eggs which are transported to hatcheries, sometimes on the same site, where they are incubated in batches until hatch. After hatch, chicks are sold as 'day-old chicks' to finishers where they are grown on until slaughter at around 42 days old. This separation of chicks from maternal contact also delays colonisation of the gut by normal commensals. Instead, chicks are first colonised by environmental bacteria in hatcheries and during transport which may be possible pathogens such as *Clostridium perfringens* or *Escherichia coli* (Craven *et al.*, 2003; Jurburg *et al.*, 2019; Stanley *et al.*, 2014*a*). Poultry flocks are particularly vulnerable to enteric pathogens. While interventions such as improved biosecurity and vaccination can reduce pathogen burden, some pathogens such as *Campylobacter* are widely present within the UK chicken population (Veterinary Record, 2015). Prophylactic use of antibiotics in the poultry industry was widely adopted as a growth promoter with a secondary effect of facilitating enteric pathogen control and reducing production losses (Dibner and Richards, 2005). However, the indiscriminate use of antibiotics has led to a rise in antimicrobial resistance. In order to combat this threat to public health, the European Union enacted a

ban on the use of antibiotics as growth promoters in 2006 (Castanon, 2007). As a result, this crutch of the poultry industry must be replaced with alternatives.

Manipulation of the intestinal microbiota provides one such alternative. Many efforts to alter the microbiome have focused on the introduction of probiotics via feed or water to growing and adult chicks. However, a growing body of evidence suggests that the ability to influence microbiome composition decreases with age as a stable microbial community is established (Ballou *et al.*, 2016). Questions remain over the optimal timing and delivery mechanism for microbiota interventions. Until recently, the embryonic gut was thought to be sterile. With increasing use of molecular techniques this assumption of sterility has been challenged with some evidence showing the presence of bacteria in the embryonic gut (Pedroso *et al.*, 2016; Ilina *et al.*, 2016). For example, viable bacteria were detected in the caecal tissue from embryos at 18 and 20 d.i using fluoresence in situ hybridisation (Pedroso *et al.*, 2016). Bacterial DNA from Enterobacteriaceae, Actinomycetales, Bifidobacteriales and Lachnospiraceae was detected using T-RFLP of the entire gastrointestinal tract of chicken embryos (Ilina *et al.*, 2016) raising the possibility that *in ovo* microbial colonisation occurs in proximal parts of the gastrointestinal tract as well as the caecum. However, scepticism of such results is not unwarranted as low microbial biomass samples are known to be prone to contamination leading to false positive results and inflated microbial diversity (Karstens *et al.*, 2019). The presence of bacteria within embryos and eggs would pose a question as to their origin. Vertical transmission is one possibility but considered unlikely (Barrow, 1994). Germ-free chicks can be derived by sterilising the eggshell immediately after lay and rearing in an isolator indicating that vertical transmission would be an uncommon route of colonisation for normal microbiota (Drew *et al.*, 2003; Sylte *et al.*, 2017). This suggests that the principal entry route for bacteria would be penetration of the eggshell and subsequent egg defences. Most studies focus on the ability of *Salmonella* and other bacteria of public health importance to translocate from the eggshell to the embryo although one study does demonstrate that other bacterial taxa are able to penetrate the eggshell (De Reu *et al.*, 2006). While these findings demonstrated that penetration of the eggshell is possible by certain bacterial taxa, it cannot be taken as evidence that microbes on the egg

surface are able to traverse the albumen and successfully colonise the embryonic gut. An aim of this study was to detect bacteria within the embryonic gut and to resolve whether a selection of commensal bacteria applied to the egg surface during incubation would be detected in the embryonic gut.

This study also aimed to investigate the effect of a topical application of adult caecal content on the development of the chicken intestinal microbiota and identify which bacterial taxa can be transplanted to chicks. Altering the microbiota of chicks after hatch is not a new idea. Since the 1970s, research has been conducted into the effectiveness of competitive exclusion cultures (CECs), usually anaerobically cultured bacteria from adult caecal contents, in reducing *Salmonella* infection in chicks (Rantala and Nurmi, 1973). With the observation that competitive exclusion was effective only when administered before *Salmonella* challenge (Seuna, 1979) the aim became to administer the probiotic as close to hatch as possible. The first report of *in ovo* administration of a probiotic came from Cox *et al.* (1992) who injected an undefined CEC into the air cell of 17 d.i eggs. This treatment conferred a greater resistance to *Salmonella* Typhimurium. Despite this early success, further results from injecting CECs into eggs have been variable with reports of reduced hatchability and early mortality with increased disease resistance falling short of antibiotic controls (de Oliveira *et al.*, 2014; Meijerhof and Hulet, 1997; Pedroso *et al.*, 2016; Yamawaki *et al.*, 2013). As such, it is worth questioning whether injection is the best delivery method for CEC products. Prior to disinfection at hatcheries, which aims to reduce the abundance of pathogenic bacteria which can reduce hatchability and chick performance, the egg has a surface microbiota similar to the composition of the caecal microbiota (Olsen *et al.*, 2017). A topical application of adult caecal bacteria may more accurately replicate the environment in which chickens and their commensals co-evolved where a sitting hen would regularly replenish the surface bacteria of the egg. Additionally, a spray application would remove the issue of hatchability caused by injecting probiotics into eggs. A previous experiment has explored the ability of a topical application of diluted adult caecal content to affect microbiota development but little analysis was conducted to determine which ASVs were successfully transplanted from the donor material to recipient chicks (Donaldson *et al.*,

2017). This is an important question in terms of developing interventions for commercial use. Regulators in several countries are unlikely to approve a treatment of unclassified bacteria sourced directly from adult chicken caecal content. Identifying bacterial taxa that are likely to be successfully transplanted by topical application is the first step towards creating an effective topical probiotic which is acceptable to regulators.

3.2 Materials and Methods

Two separate trials were conducted, a pilot experiment and repeat experiment. While the methods used between them were similar, some differences were applied to the repeat experiment based on the results from the pilot experiment. All methods are described for the pilot experiment and any deviations from this protocol further discussed for the repeat experiment.

3.2.1 Animals and housing

Pilot Experiment

61 Ross 308 eggs were purchased from a local hatchery (Annyalla Chicks, Wrexham). Hatchery eggs were disinfected daily during storage using a fog application of Virocid (Cid Lines), a disinfectant based on quaternary ammonium, glutaraldehyde and isopropanol. Eggs at the hatchery are disinfected further using formaldehyde fumigation before being set. On arrival at the experimental housing, 5 eggs were selected for sampling at 0 d.i. The remaining 56 eggs were divided into a treatment group and a control group of 28 eggs each. Each group was housed in different incubators in different rooms. A biosecurity protocol was implemented whereby the control group was handled first to avoid transfer of environmental bacteria from the treatment to the control group. Eggs were incubated at 37.5°C for 21 days. The eggs were candled at 7 d.i to assess viability. In both groups, 8 eggs were removed as no embryonic development had occurred. Five eggs from each group were removed for sampling at 18 d.i. The remaining 15 eggs in each group were left to hatch. 15 and 14 chicks hatched from the treatment and control groups respectively. Chicks

were left in the incubators until dry before being transferred to brooder pens with a wood shaving substrate. Water and feed were provided *ad libitum* by a drinker and feeder present in each brooder. Chicks were fed the same vegetable protein based starter diet as described in Chapter 2. Nutritional composition of the starter and grower diets is displayed in Table 2.1 and a full list of ingredients provided in Table A.1. Seven chicks from each group were sampled at 3 d.p.h with the remaining 8 treated and 7 control chicks sampled at 7 d.p.h. No unexpected deaths occurred in either group over the course of the experiment.

Repeat Experiment

56 Ross 308 eggs were purchased from a local hatchery (Annyalla Chicks, Wrexham). Eggs underwent the same disinfection procedure at the hatchery as described for the pilot experiment. On arrival at the experimental housing, the 56 eggs were divided between two incubators of 28 eggs each. The day that incubation started was defined as 0 d.i. Both incubators were housed in the same room. Eggs were incubated at 37.5°C for 22 days. The eggs were candled at 7 d.i to assess viability. Eggs began to hatch at 20 d.i with 6 chicks hatching on 20 d.i, 21 on 21 d.i and 17 on 22 d.i giving a total of 44 chicks. After hatch, chicks were left in the incubators until dry before being transferred to brooder pens with a wood shaving substrate. At 22 d.i, 40 day old chicks were purchased from the same hatchery. These chicks were the control group and were housed separately from treated chicks. Water and feed were provided *ad libitum* by a drinker and feeder present in each brooder. Chicks were fed the same vegetable protein based starter diet for the duration of the experiment. Five chicks from each group were sampled on the same day the control chicks were brought to the housing (defined as 0 d.p.h). Ten chicks from each group were sampled at 3, 7 and 14 d.p.h. Two chicks from the treatment group died unexpectedly during the experiment, one at 1 d.p.h and another at 6 d.p.h. The cause of death was not determined although a preliminary gross post mortem examination revealed peritonitis and perihepatitis consistent with early opportunistic bacterial infection.

All experimental protocols were approved by the University of Liverpool's Animal Welfare and Ethical Review Body and conducted in accordance with the Animals (Scientific

Procedures) Act 1986.

3.2.2 Treatment

Entire caecal contents were collected from healthy 42 day old chickens from three different breeds (Ross 308, Hubbard JA87 and Cobb 500) described in Chapter 2. 200mg of caecal contents from five individuals of each breed were pooled and DNA extracted for sequencing. The remaining caecal contents were stored at -20°C for 14 months. Before experimental work began, caecal contents from Ross, Cobb and Hubbard birds were defrosted, mixed and diluted 1:20 in sterile phosphate buffered saline. Aliquots of 5ml of diluted caecal content were prepared and frozen at -20°C for use as treatments. Treatment group eggs were sprayed at 2, 7, 14 and 18 d.i in both trials. The diluted caecal contents was defrosted at room temperature and loaded into a 10ml spray bottle. Eggs were sprayed evenly at a distance of 10cm ensuring all eggs received at least two sprays until the 5ml of diluted caecal content had been used.

3.2.3 Sample collection

Pilot Experiment

Samples were taken from eggs at 0 d.i. To minimise the risk of contamination, eggs were sprayed with 70% ethanol and left for 10 minutes before being wiped clean. Samples were taken in as sterile an environment as possible. All samples were taken inside an exclusion cabinet and sterile gloves were worn and changed between eggs. An electric rotary tool (Dremel 3000) was used to cut through the egg shell without penetrating the shell membranes. A sterile scalpel was used to cut the shell membrane to remove the top of the egg shell and reveal the yolk. Sterile needles and syringes were used to sample from the albumen and the yolk.

At 18 d.i, samples were taken from five embryos. Embryos were killed by refrigerating the egg at 3°C for four hours. The egg shell was opened as previously described. A sample of amniotic fluid was taken using a sterile needle and syringe. The embryo was removed from

the egg, placed in a sterile petri dish and placed under a stereomicroscope for dissection. Using a sterile scalpel and forceps, the coelom was opened to reveal the gastrointestinal tract which was removed. The duodenum, jejunum and ileum were stored together with both caeca stored in a separate container. Finally, the brain was removed using a new sterile scalpel to be used as a control for contamination should bacterial DNA be recovered from the gastrointestinal tract.

Further samples were taken at 3 and 7 d.p.h. Chicks were euthanased by cervical dislocation. To sample chicks, the abdomen was sprayed with 70% ethanol. Skin incisions were made to expose the sternum which was then reflected to give good access to the coelom. The gastrointestinal tract was removed carefully to avoid external contamination. The ileum, defined as the intestinal segment from Meckel's diverticulum to the ileocaecocolic junction; and both caeca were removed and stored in separate containers. Samples for DNA extraction were snap frozen in liquid nitrogen and stored at -20°C.

Repeat Experiment

The same sampling protocol was used as for the pilot experiment with chicks sampled at 0, 3, 7 and 14 d.p.h. After euthanasia chicks were weighed and their body weight recorded in grams. Additionally, tissue samples from the caecal tonsils, identified as the proximal section of the caecum, and the ileum were taken. One caecal tonsil and a section of ileum were fixed in 4% paraformaldehyde solution for histological examination. The other caecal tonsil was fixed in optimal cutting temperature (OCT) compound (CellPath, UK) on a cork plate and snap frozen in liquid nitrogen. Samples fixed in paraformaldehyde were stored at 4°C, samples for DNA extraction were stored at -20°C and samples fixed in OCT compound were stored at -80°C.

3.2.4 DNA extraction

DNA was extracted from each sample using Zymobiomics DNA MiniKits (Cambridge Bioscience, UK) according to the manufacturer's instructions. DNA was extracted from 250µl of liquid samples (albumen, yolk and amniotic fluid). For tissue samples (ileum

and caecum), a 200mg section of intestinal tissue along with content was used for DNA extraction. This section was cut longitudinally and transversely using a sterile scalpel blade to expose the mucosa and luminal contents to beat-beating. Both liquid and tissue samples underwent a bead-beating step using a Qiagen TissueLyser at 30Hz for 10 minutes. At each extraction, two controls were included: a blank extraction kit to control for contamination and 75µl of Zymobiomics Standard Bacterial Community (Cambridge Bioscience, UK) to control for variations in DNA extraction efficacy. Extracted DNA was quantified using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies).

3.2.5 PCR to detect bacterial DNA

The detection of bacterial DNA in egg and embryonic samples was performed by PCR amplification of bacterial 16S rRNA genes. Purified DNA from egg and embryonic samples from the pilot experiment was used as the template in a PCR mixture composed of 5µl of 5x FIREPol Master Mix Ready to Load (Solis BioDyne, Estonia), 1µl of each primer, 17µl of purified water and 1µl of DNA template. A primer pair spanning the V4 region of the 16S rRNA gene (515F: TGCCAGCMGCCGCGGTAA, R806: GGACTACHVGGGTWTCTAAT) was used (Caporaso *et al.*, 2011). DNA extracted from the Zymobiomics Standard Bacterial Community (ZSBC), which contains approximately $1.4x10^{10}$ cells/ml, was used as a positive control. Thermal cycling consisted of an initial cycle of 95°C for 5 min, 30 cycles of 95°C for 30s, 55°C for 45s and 72°C for 40s followed by a final cycle of 72°C for 40s. The presence of PCR products was confirmed by electrophoresis using a 1.0% agarose gel containing ethidium bromide. To exclude the possibility that negative results were due to PCR inhibitors present within samples, 9µl of each sample was spiked with 1µl of DNA extracted from the ZSBC and submitted for PCR amplification. To determine the sensitivity of the PCR assay, DNA extracted from the ZSBC was diluted to include the equivalent of DNA extracted from 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 bacterial cells.

3.2.6 Illumina MiSeq sequencing

Extracted DNA was sent for paired-end sequencing of the 16S rRNA gene at the Centre for Genomic Research (University of Liverpool) as described in Chapter 2.2.4. Raw sequence reads are available in the NCBI Sequence Repository Archive under BioProject PRJNA517619.

3.2.7 Amplicon sequence variant identification and taxonomy assignment

QIIME2 version 2019.1.0 was used for analysis of the Illumina data (Bolyen *et al.*, 2019) and conducted as described in Chapter 2.2.5

3.2.8 Data analysis

Diversity analyses, production of taxa plots and identification of differentially abundant taxa was conducted as described in Chapter 2.2.6 with some modifications. Alpha and beta diversity analyses were performed at a sampling depth of 5,000 for caecal samples and 4,800 for ileal samples using the alignment (Katoh and Standley, 2013), phylogeny (Price *et al.*, 2010) and diversity (https://github.com/qiime2/q2-diversity) plugins. Beta diversity was calculated with an unweighted and weighted UniFrac metric.

3.2.9 Statistics

Samples from the ileum and caecum were analysed separately. For statistical analysis, samples were grouped according to age, treatment group and experiment creating 13 different groups for comparison in each organ system. Sample groups are identified using abbreviations in which the first letter corresponds to the experiment (P, pilot; R, repeat), the second letter corresponds to the treatment (C, control; T, treated), and the numbers correspond to the time point (0, 3, 7, or 14 d.p.h). Transplant material is identified by the abbreviation TRPL. The number of samples from each organ system per group varied (PC3, n=7; PT3, n=7; PC7, n=7; PT7, n=8), samples from the repeat experiment from control (C) and treated (T) chicks at 0, 3, 7 and 14 d.p.h (RC0, n=5; RT0, n=5; RC3, n=7;

RT3, n=7; RC7, n=7; RT7, n=7; RC14, n=7; RT14, n=7) and transplant material (TRPL, n=3). Alpha diversity was compared between groups using a pairwise Kruskal-Wallis test with an FDR correction. An ANOSIM test was used to identify metadata categories which were significantly affected beta diversity. The average distance from samples in each group to TRPL samples was compared using an independent Students t-test to find which group was closest to TRPL samples. Gneiss analysis (Morton *et al.*, 2017) was used to identify taxa which were differentially abundant between treatment and control groups in the pilot and repeat experiments separately. First, the ASV table was filtered to exclude transplant samples and low abundance ASVs. In caecal samples, the count threshold for exclusion of ASVs was set at the first quartile to exclude the lowest 25% of ASVs by total frequency across all caecal samples. Due to the high number of low abundance ASVs present in ileal samples the count threshold for exclusion of ASVs was set at the median to exclude the lowest 50% of ASVs by total frequency across all ileal samples. Otherwise, Gneiss analysis was conducted as described in Chapter 2.2.6 Results were visualised through a regression summary, dendrogram heatmaps and balance taxonomies to identify ASVs which were differentially abundant in treated and control groups. Based on this analysis ASVs were divided into three groups: ASVs with a higher abundance in treated samples, ASVs with a higher abundance in control samples and ASVs with no differential abundance between groups. The results of this analysis were used to select taxa for further analysis using quantitative PCR.

3.2.10 Identifying features transplanted from the treatment

ASVs present in an unfiltered ASV table of TRPL samples were defined as present in the transplant material. The same ASV table used for Gneiss analysis was used to compile a list of ASVs present in each sample group. Once lists of ASVs were compiled for the transplant and sample groups, intersections between sets of ASVs were visualised using UpSet (Lex *et al.*, 2014). ASVs which were present only in the transplant were removed to facilitate visualisation of other intersections. Based on their presence in intersections ASVs were classified as successfully transplanted, possibly transplanted or environmental.

ASVs were classified as successfully transplanted if they were present in the transplant and in treated chicks at least one time point before control chicks. ASVs were classified as possibly transplanted if they were present in the transplant and in both treated and control chicks at the same time point. ASVs were classified as environmental if they were present in the transplant and present in only control chicks or present in control chicks before treated chicks. Any ASV not present in the transplant was classified as environmental. A chi-square test of independence was performed to examine the relationship between ASVs identified as differentially abundant between treatment groups and those defined as successfully transplanted, possibly transplanted or environmental using Python's scipy module (Jones *et al.*, 2001). The taxonomy of ASVs classified as successfully transplanted, possibly transplanted and environmental was compared to that of ASVs identified as more abundant in treated chicks, more abundant in control chicks and not differentially abundant with a hybrid Sankey diagram created using sankeyview (version 1.7.7) (Lupton and Allwood, 2017).

3.2.11 Quantitative PCR

Taxa were selected for further testing using quantitative PCR based on results from Gneiss analysis. Primers were selected from the literature or constructed using the method described in Chapter 2.2.7. Primers used are displayed in Table 3.1. The real-time quantitative PCR assay was conducted on a 1:10 solution of extracted DNA using a Rotor-Gene Q PCR machine (Qiagen) and PrecisionPLUS qPCR master mix (Primer Design, UK). The V4 region of the 16S rRNA gene was used as a reference gene. Rotor-Gene Q software (version 2.3.1.49) was used to produce melting curves and identify the cycle threshold (Ct), the point at which fluorescence above the background level is detectable. Each sample was run in triplicate with an averaged Ct used in further analysis. The ΔCt, defined as the difference between the Ct value for taxa specific primers and the Ct value for the reference gene, was calculated for each sample. Results were expressed as 40 – ΔCt. Results were compared between treatment groups at equivalent time points and experiments using an independent Student's t-test. An FDR correction for multiple tests was applied to p-values and the

significance threshold was set at 0.05. Amplification of DNA in one PC3 sample failed in all reactions. As a result, this sample was excluded from quantitative PCR analysis.

Target Taxa	Primers	Amplicon Size (b.p.)	Reference
Domain *Bacteria* (targets V4 region)	F: TGCCAGCMGCCGCGGTAA R: GGACTACHVGGGTWTCTAAT	254	(Caporaso *et al.*, 2012)
Clostridium	F: TGCCAGCMGCCGCGGTAA R: GGACTACHVGGGTWTCTAAT	131	(Amit-Romach *et al.*, 2004)
Enterobacteriaceae	F: GTGCCAGCMGCCGCGGTAA R: GCCTCAAGGGCACAACCTCCAAG	429	(Smith *et al.*, 2014*b*)
Candidatus *Arthromitus*	F: GATGCGTAGGCGGTTGAGTA R: GGGTTTCTAATCCTGTTTGCTCC	90	This study
Clostridium cluster IV	F: TTACTGGGTGTAAAGGG R: TAGAGTGCTCTTGCGTA	580	(Van Dyke and McCarthy, 2002)
Clostridium cluster XIVa&b	F: AAATGACGGTACCTGACTAA R: CTTTGAGTTTCATTCTTGCGAA	438-441	(Matsuki *et al.*, 2002)

Table 3.1: Primer pairs used for quantitative PCR

3.2.12 Histology

Haematoxylin and eosin staining

Tissue fixed in 4% paraformaldehyde solution was examined histologically to identify differences in morphological development of the ileum and caecal tonsil between treated and control chicks. Four sections of ileum and four sections of caecal tonsil from each chick underwent tissue processing using a Tissue-Tek vacuum infiltration processor overnight before being embedded in paraffin (Ultraplast premium embedding medium, Solmedia). 4µm paraffin sections were cut on a Leica RM2125 RT microtome, floated on a waterbath and placed on colour slides (Solmedia, MSS54511YW). For haematoxylin and eosin (H&E) staining slides were dewaxed in xylene and rehydrated through descending grades of ethanol (100%, 96%, 85%, 70%) to distilled water before being stained in haematoxylin (5mins), blued in tap water for 5 minutess and stained in eosin (2 minutess). Slides were then dehydrated through 96% and 100% ethanol to xylene and cover slipped using DPX (Thermo Scientific, Lamb/DPX). Haematoxylin (Atom Scientific, RRBD61-X) and eosin (TCS,

HS250) solutions made up in house.

H&E stained tissue sections were examined by light microscopy (Nikon Eclipse 80i) with a Leica DMC 4500 digital camera attachment (Leica Microsystems, Switzerland). Images were viewed and measurements taken using Leica Application Suite X software. Sections were assessed for suitability based on orientation of tissue samples. Villus height, villus width and epithelial cell height were recorded in transverse ileal sections where entire villi could be visualised to the lamina propria. In such sections, the height and width of five villi with an intact lamina propria was measured. Villus height was defined as the distance from the villus tip to the villus-crypt junction. Villus width was measured at the widest section of the villus. Epithelial cell height was measured at the villus tip and was defined as the distance from the distal point of the microvilli to the basement membrane. Measurements were expressed as a mean for each bird.

Mitotic figure counts in the ileum and caecal tonsil were used as an indication of intestinal villus development (Yamauchi *et al.*, 1993; Yamauchi, 2007). All orientations of tissue were included for mitotic counts where crypts were adjacent to the lamina propria and muscular layers. Mitotic figures in crypts within one high power field (400x) of the lamina propria were counted. Mitotic figures were identified as strongly basophilic and homogenous nuclei with care taken to count cells in the late stages of division as a single mitotic figure. The length of lamina propria over which mitotic figures were counted was measured and results expressed as number of mitotic figures per 100µm. Results were expressed as a mean for each bird. Results were compared between treatment groups using Students t-test implemented in the scipy (version 1.1.0) Python module (Jones *et al.*, 2001). A Benjamini-Hochberg false discovery rate correction implemented in the statsmodels (version 0.9.0) Python module (Seabold and Perktold, 2010) was applied to account for multiple tests. Results were compared between age groups using a one-way analysis of variance test with a post-hoc Tukey HSD test when significant differences were identified.

During the analysis, it was noted that some samples had large aggregates of bacterial cells within the crypts of the caecal tonsil. In order to ascertain whether the presence of bacteria in the caecal tonsil crypts was associated with age or treatment group, slides were

re-examined. Samples were classified as positive if bacteria were observed in more than one crypt and in at least two sections.

Immunostaining

Serial 7.5µm thick sections of caecal tonsil tissue frozen in OCT were cut using a cryostatic microtome. Four sections of caecal tonsil from each bird were mounted on poly-l-lysine coated slides (VWR International, UK) and fixed in acetone for 10 minutes. Immunostaining was performed on a Dako Autostainer Link 48 using Envision™ FLEX reagents. Following a buffer rinse tissue sections underwent a peroxidase block for 5 minutes (Agilent, SM801) before being incubated for 20 minutes with mouse monoclonal antibodies against chicken CD4, CD8α, CD8β, γδTCR and Bu1 (B cells and subsets of monocytes and macrophages), antigens (Cambridge Bioscience Ltd, 8210-01, 8220-01, 8280-01, 8230-01 and 8395-01 respectively). The antibodies CD4 (1:200), CD8α(1:200), CD8β(1:1000), γδTCR (1:100) and Bu1 (1:400) were diluted in Envision™ FLEX Antibody Diluent (Agilent, K8006). Antibody binding was detected using the labelled polymer Envision™ FLEX/HRP (Agilent, SM802) for 20mins and the reaction visualised using the substrate-chromogen FLEX DAB+Sub Chromo (Agilent, DM827 SM802). Tissue sections were counterstained for 5mins in Envision™ FLEX Haematoxylin (Agilent, K8008), washed in deionized water and dehydrated through increasing grades of ethanol (85%, 96%, 3x 100%) before clearing in xylene and mounted as per H&E staining above. All intermediate buffer washes between reagents used Envision™ FLEX Wash Buffer (K8007). Stained tissue sections were examined using the same apparatus as described for H&E stained tissue. Quantification of cell abundance between treated and control chicks was performed by counting cells in photographs taken at a magnification of 200 with each field of view representing an area of 142,000µm^2. Five photographs for each bird taken randomly from serial sections were used. Results were expressed as a mean for each bird. Student's t test was used to identify significant differences in cell abundance between treatment groups.

3.3 Results

3.3.1 Sequencing effort

A total of 22,103,523 reads were obtained from 182 experimental samples submitted for sequencing. After filtering, merging of paired reads and chimera removal, a total of 15,022,950 reads remained (68% of the original total) giving a mean of 82,544 reads per sample. The median number of reads per sample was 92,218.

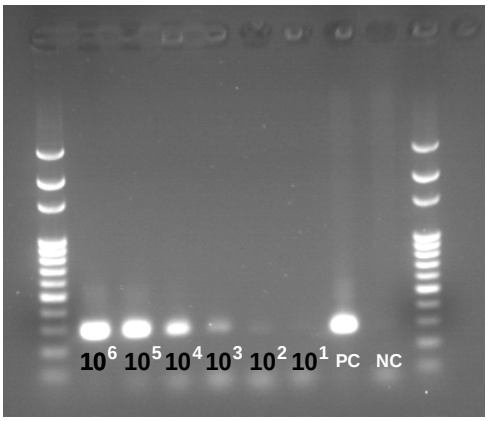
3.3.2 Detection of bacterial 16S rRNA genes in embryonic samples

Amplification of standard dilutions revealed that the PCR assay was able to clearly detect 10^3 bacterial cells in a sample (Figure 3.1). No positive amplification of bacterial 16S rRNA genes was detected in any embryonic or egg sample at either 0 or 18 d.i. Amplicons were detected in positive control samples and all spiked samples indicating that the absence of amplicons in other samples was not due to PCR failure. This result indicates that no significant population of bacteria was present in the embryonic gut at 18 d.i.

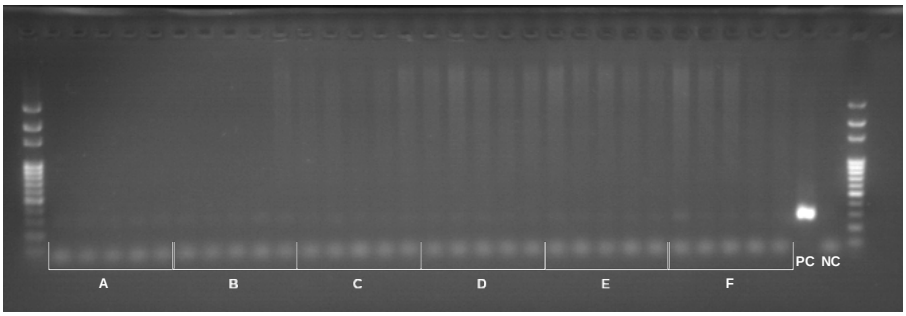
3.3.3 Body weight

Repeat Experiment

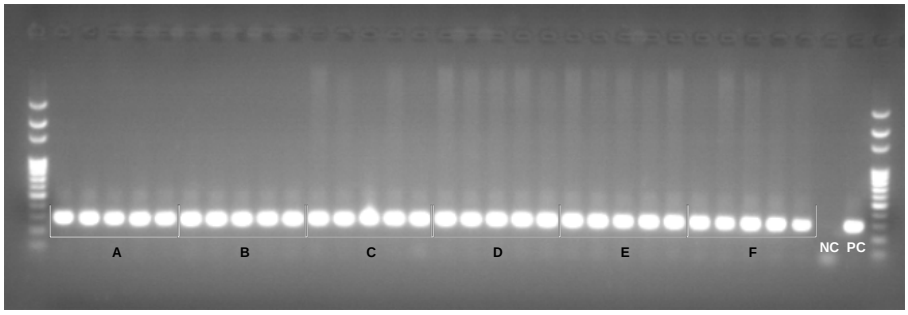
The mean body weight of treated and control chicks was compared using Students t-test. No significant differences between groups were found at 0 (treated: M = 46g, SD = 5.83; control: M = 48.4g, SD = 6.65; conditions: t = -0.5, p = 0.6), 7 (treated: M = 135g, SD = 19.0; control: M = 132g, SD = 17.1; conditions: t = 0.35, p = 0.7) and 14 (treated: M = 358.5g, SD = 44.8; control: M = 322.5g, SD = 39.8; conditions: t = 1.8, p = 0.13) d.p.h. However, there was a significant difference in body weight between groups at 3 d.p.h (treated: M = 77g, SD = 7.48; control: M = 65g, SD = 5.0; conditions: t = 4.0, p = 0.002).



(a) Standard dilutions of bacterial DNA from 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 bacterial cells including a positive (PC) and negative control (NC).



(b) DNA extracted from amniotic fluid from control (A) and treated (B) eggs; duodenum, jejunum and ileum from control (C) and treated (D) embryos and caeca from control (E) and treated (F) embryos including a positive and negative control.



(c) Samples spiked to include bacterial DNA along with a negative and positive control.

Figure 3.1: Gel images showing PCR products from embyronic samples

3.3.4 Alpha diversity

A sampling depth of 5000 for caecal samples resulted in no samples excluded from the analysis. One sample, a treated sample from 3 d.p.h in the repeat experiment, was excluded from ileal analysis using a sampling depth of 4800.

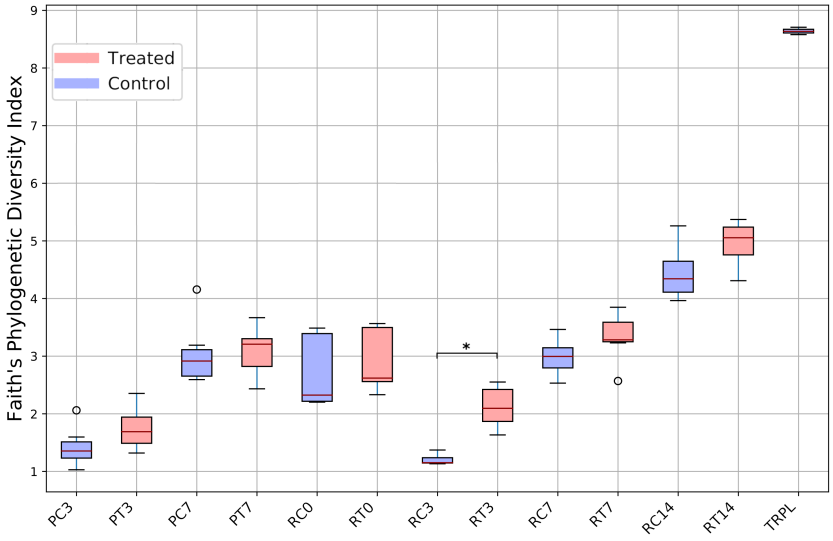
Caecum

The alpha diversity measured by FPD and SD indices of each sample group is displayed in Figure 3.2 with the significance of pairwise Kruskal-Wallis tests comparing alpha diversity between sample groups displayed in Figure B.1.

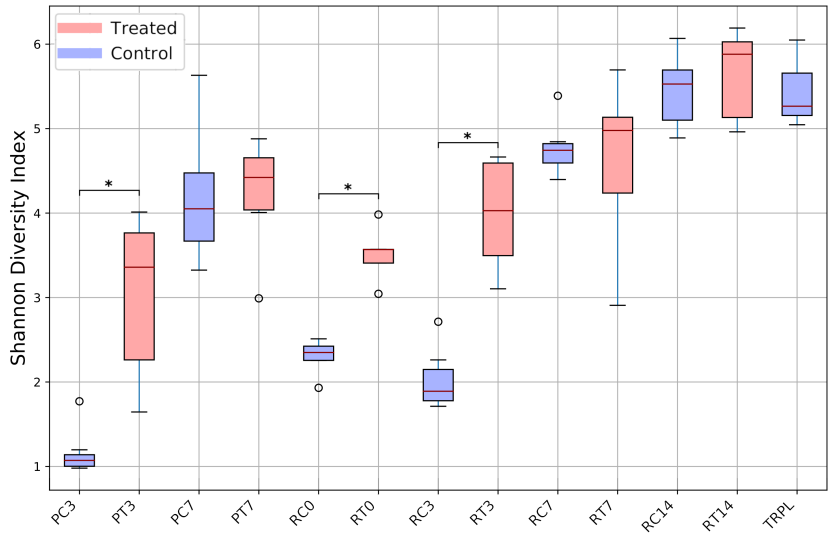
Across all experimental groups alpha diversity increased significantly with age using both diversity indices with some exceptions. There was no significant increase in SD of treated or control chicks between 0 and 3 d.p.h (treated: $H = 1.91$, $p = 0.19$; control: $H = 2.38$, $p = 0.15$) or in treated chicks between 3 and 7 d.p.h during the repeat experiment ($H = 2.16$, $p = 0.17$). FPD decreased significantly in treated and control chicks between 0 and 3 d.p.h (treated: $H = 5.55$, $p = 0.03$; control: $H = 8.1$, $p = 0.008$).

Treatment with an adult-derived microbiome resulted in a significantly higher SD when compared to control chicks at 0 d.p.h (Repeat Experiment: $H = 6.82$, $p = 0.017$) and 3 d.p.h (Pilot Experiment: $H = 9.02$, $p = 0.009$; Repeat Experiment: $H = 9.8$, $p = 0.006$) but not 7 and 14 d.p.h. There were significant differences in SD between TRPL samples and samples taken at 0 and 3 d.p.h as well as PT7 samples. The FPD of treated chicks was not significantly higher than control chicks at 0 d.p.h in the repeat experiment ($H = 1.84$, $p = 0.21$) or 3 d.p.h in the pilot experiment ($H = 2.55$, $p = 0.14$). However, there was a significantly higher FPD in treated chicks at 3 d.p.h in the repeat experiment ($H = 9.8$, $p = 0.006$). FPD of TRPL samples was significantly higher than all other groups. Overall, these results show that while the taxonomic diversity of treated chicks wasn't necessarily higher than control chicks, there was a significantly greater evenness in treated chicks at 0 and 3 d.p.h.

Control chicks at 3 d.p.h had significantly higher SD in the repeat experiment than



(a) FPD Index



(b) SD Index

Figure 3.2: Alpha diversity of caecal samples in the pilot and repeat experiments

Significant differences between treated and control chicks from the same time point are indicated (*).

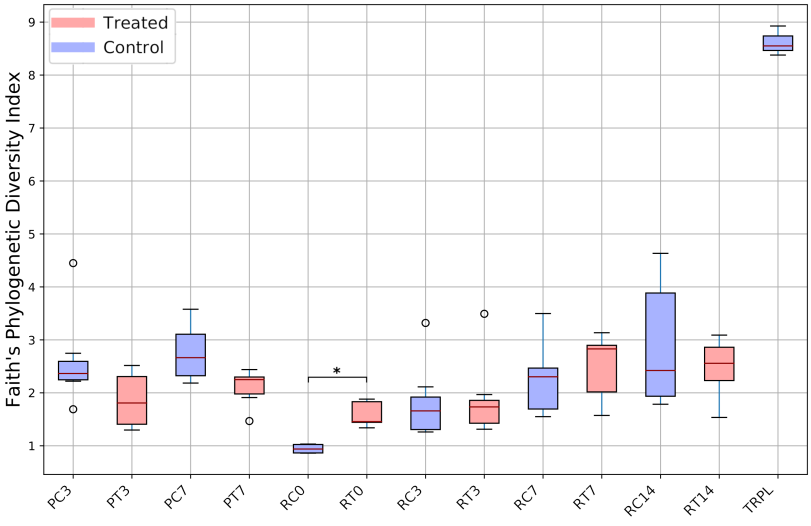
the pilot experiment (H = 8.27, p = 0.01). There were no further significant differences in alpha diversity between equivalent groups from the pilot and repeat experiments.

Ileum

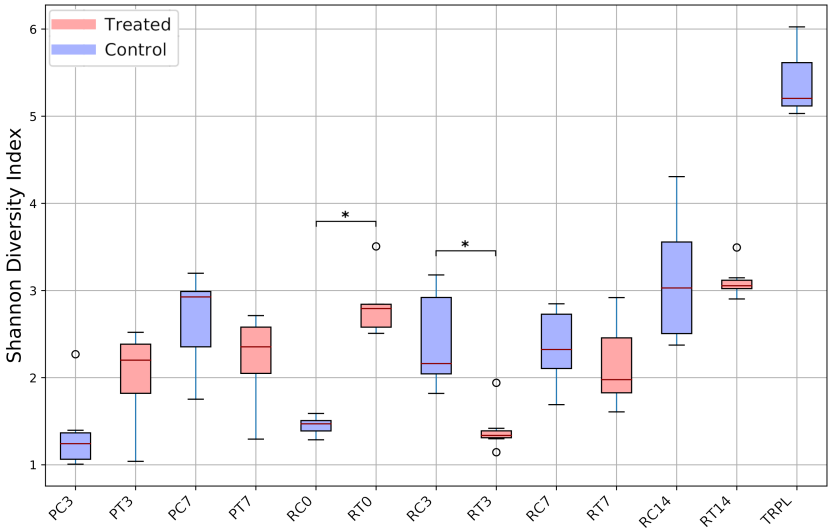
The alpha diversity of each sample group is displayed in Figure 3.3. Unlike in the caecum, there was not a clear pattern of increasing alpha diversity with age. In the pilot experiment, SD increased between 3 and 7 d.p.h in control chicks but not treated chicks (treated: H = 0.6, p = 0.5; control: H = 9.2, p = 0.02). FPD was not significantly different between consecutive time points in either treated or control chicks. In the repeat experiment, SD increased between 0 and 3 d.p.h in control chicks (H = 8.1, p = 0.02) but not between subsequent time points. In contrast, treated chicks showed a decrease in SD between 0 and 3 d.p.h (H = 8.1, p = 0.02) with subsequent significant increases in SD between 3 and 7 d.p.h (H = 7.55, p = 0.02) and 7 and 14 d.p.h (H = 9.02, p = 0.02). FPD was increased between 0 and 3 d.p.h in control chicks (H = 8.1, p = 0.03) but there were not differences in FPD between consecutive time points in the treated group.

No significant differences in SD or FPD were noted between treatment groups in the pilot experiment. In the repeat experiment, significant differences in SD were observed between treated and control chicks at 0 and 3 d.p.h. At 0 d.p.h, treated chicks had significantly higher SD (H = 6.81, p = 0.02) while at 3 d.p.h control chicks had significantly higher SD (H = 9.02, p = 0.02). The SD of the transplant material was significantly higher than all sample groups. FPD was only significantly higher in treated compared to control chicks at 0 d.p.h (H = 6.81, p = 0.05).

A significant difference in SD between equivalent groups from the pilot and repeat experiments was only observed between control chicks at 3 d.p.h with a higher SD found in the repeat experiment (H = 6.86, p = 0.02). No significant differences in FPD between equivalent groups were found.



(a) FPD Index



(b) SD Index

Figure 3.3: Alpha diversity of ileal samples in the pilot and repeat experiments

Significant differences between treated and control chicks from the same time point are indicated (*).

3.3.5 Beta diversity

Caecum

When measured with an unweighted UniFrac metric, the factor 'Age' had the largest effect on beta diversity measured by unweighted UniFrac distance (ANOSIM test statistic = 0.78, p = 0.001), followed by 'Treatment' (ANOSIM test statistic = 0.13, p = 0.001) and 'Experiment' (ANOSIM test statistic = 0.10, p = 0.012). A PCoA plot showed clustering of samples by group (Figure 3.4a). The average unweighted UniFrac distance between groups is displayed in Figure B.2. When measured with a weighted UniFrac metric, the factor 'Age' had the largest effect on beta diversity (ANOSIM test statistic = 0.40, p = 0.001), followed by 'Experiment' (ANOSIM test statistic = 0.16, p = 0.002) and 'Treatment' (ANOSIM test statistic = 0.13, p = 0.001). A PCoA analysis showed clustering of samples by group (Figure 3.4b)

In plots of unweighted UniFrac distance, RC0 and RT0 samples tended to cluster together in the PCoA plot with the exception of one RT0 sample. PC3 and RC3 samples clustered together along with three PT3 samples. The remaining PT3 samples and all RT3 samples clustered togther and were closer to samples from later time points than PC3 and RC3 samples. At 7 d.p.h, control and treated samples from both experiments clustered together although there was a tendency for treated samples from both experiments to cluster closer to samples from 14 d.p.h. RC14 and RT14 samples formed separate clusters to each other. A similar pattern of clustering was present in plots of weighted UniFrac distance although there was no separate clustering of RC14 and RT14 samples. Instead, all samples from 7 and 14 d.p.h tended to cluster together along with RT3 samples and one PT3 sample. Additionally, PC3 and RC3 samples formed distinct clusters compared to the unweighted UniFrac distance plot.

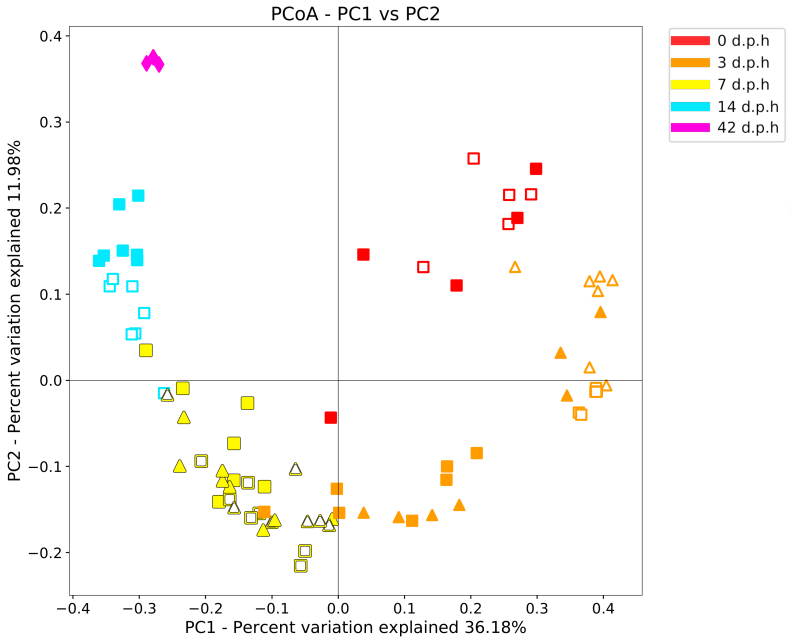
Distance between sample groups and TRPL samples gives some indication of transplant success as the unweighted UniFrac distance between similar samples is lower reflecting closer clustering of samples. At 3, 7 and 14 d.p.h treated samples were significantly closer to TRPL samples than controls (3 d.p.h: t = 16.6, p <0.001 ; 7 d.p.h: t = 7.2, p <0.001

and 14 d.p.h: t = 4.8 , p <0.001). This pattern of increased similarity of treated samples to TRPL samples in both experiments suggests that bacteria from adult caecal content successfully colonised chicks by those time points. However, the success of the treatment was not uniform between experiments as PT3 samples were further from TRPL samples than RT3 samples (0.76 c.f. 0.66).

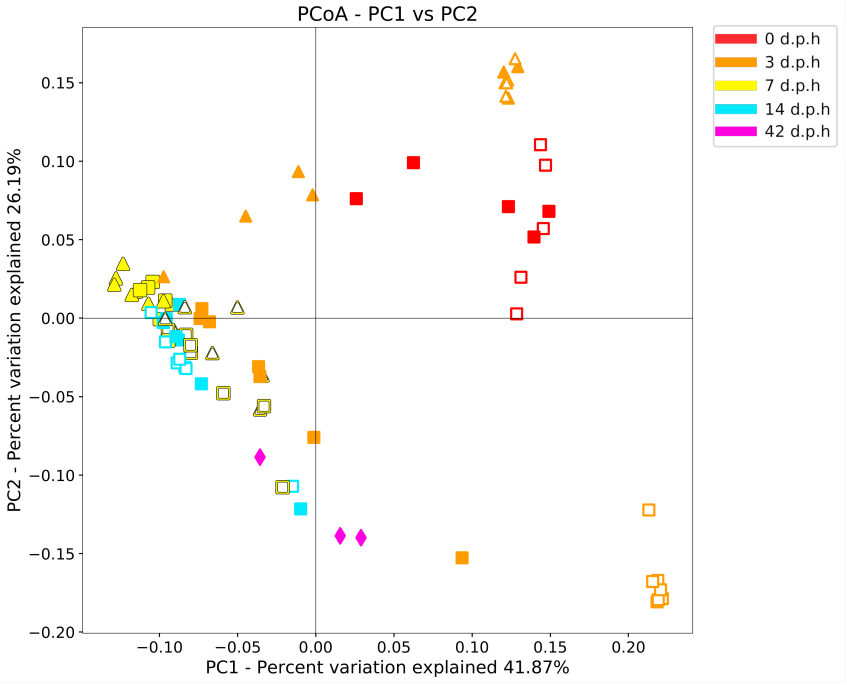
Ileum

When measured using an unweighted UniFrac metric, the factor 'Age' had the largest effect on beta diversity measured by unweighted UniFrac distance (ANOSIM test statistic = 0.42, p = 0.001), followed by 'Experiment' (ANOSIM test statistic = 0.20, p = 0.001) and 'Treatment' (ANOSIM test statistic = 0.12, p = 0.002). A PCoA plot showed clustering of samples by group (Figure 3.5a). When measured with a weighted UniFrac metric, the factor 'Age' had the largest effect on beta diversity (ANOSIM test statistic = 0.28, p = 0.001), followed by 'Experiment' (ANOSIM test statistic = 0.13, p = 0.004) and 'Treatment' (ANOSIM test statistic = 0.11, p = 0.001). A PCoA analysis showed clustering of samples by group (Figure 3.5b)

In plots of unweighted UniFrac distance, Exp2_CI00 and Exp2_TI00 samples formed separate clusters in the PCoA plot suggesting a different microbiota composition. Samples from the same experiment taken at 3 d.p.h formed separate clusters from those taken at 0 d.p.h. Samples taken at 3 d.p.h in the pilot experiment clustered with those from the repeat experiment with some separation of treated and control samples suggesting a different microbiota composition. Exp2_CI07 and Exp2_TI07 samples clustered away from samples taken at 3 d.p.h but no difference in clustering by treatment group was observed. Samples from the pilot experiment taken at 7 d.p.h clustered with those from the repeat experiment and did not show clustering by treatment group. Exp2_CI14 and Exp2_TI14 samples did not cluster separately from samples taken at 7 d.p.h suggesting that the composition of the microbiome was similar between these time points. Similar to unweighted UniFrac distance, Exp2_CI00 and Exp2_TI00 samples clustered separately in the PCoA plot of weighted UniFrac distance. Samples taken at 3 d.p.h in the pilot experiment did not cluster with



(a) Unweighted UniFrac



(b) Weighted UniFrac

Figure 3.4: Beta diversity between caecal samples from the pilot and repeat experiments

Samples from the pilot experiment (▲) and the repeat experiment (■) are shown with treated (filled) and control (no fill) samples denoted by colour fill. Transplant samples are also shown (⧫)

those from the repeat experiment with some separation of control and treated samples. From 7 d.p.h there was no further clustering by time point or treatment group.

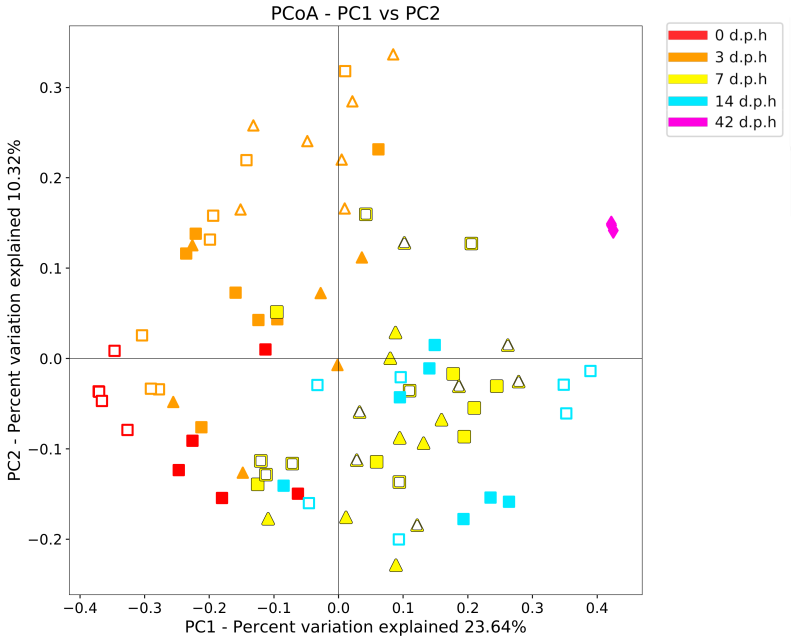
Overall, the greatest differences in beta diversity were found between treated and control chicks at 0 d.p.h. Subsequently, no differences between treated and control chicks were observed. The greatest factor influencing beta diversity was age, with shifts in microbiome composition between 0 and 7 d.p.h which were consistent between experiments and treatment group. Unlike in the caecum, the ileum showed little similarity to the transplant material. However, Exp2_TI00 samples were, on average, significantly closer to TRPL samples than Exp2_CI00 samples were ($t = 10.9$, $p < 0.001$) when beta diversity was measured using an unweighted UniFrac metric.

3.3.6 Taxonomic composition

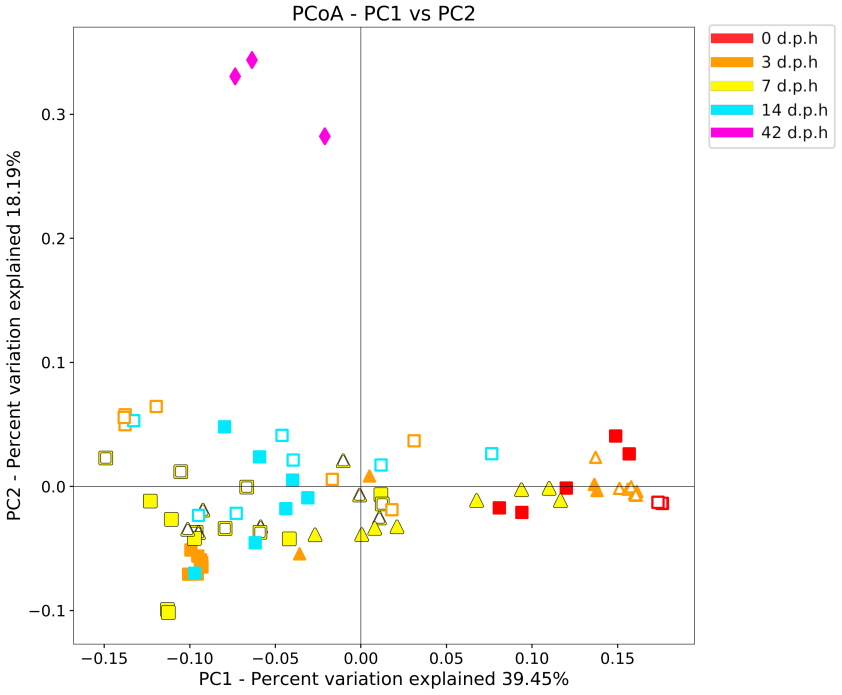
Caecum

The family of ASVs present within each sample was displayed in a taxa plot (Figure 3.6). The transplant material was mainly composed of Bacteroidaceae, Ruminococcaceae and Lachnospiraceae. Families present in the transplant material with a smaller relative abundance included Enterobacteriaceae, Lactobacillaceae, Clostridiales vadin BB60 group, Mollicutes RF39, Burkholderiaceae, Christensenellaceae, Peptococcaceae, Bifidobacteriaceae and Atopobiaceae.

In the pilot experiment, there were visible differences between the microbiome composition of treated and control chicks at 3 d.p.h. Across all PC3 samples, Clostridiaceae 1 had a relative abundance of between 95 and 99.9%. Other families with low relative abundance in this group included Bacillaceae and Paenibacillaceae. More variation was present between PT3 samples. Clostridiaceae 1 was the most abundant family in three samples, Lachnospiraceae was the most abundant family in four samples. Ruminococcaceae was present in small amounts in two samples (0.7% and 2.2%). Other families present in PT3 samples included Bacillaceae, Paenibacillaceae and Erysipelotrichaceae. A more diverse microbiome was present at 7 d.p.h in both groups. In all PC7 samples Lachnospiraceae



(a) Unweighted UniFrac



(b) Weighted UniFrac

Figure 3.5: Beta diversity between ileal samples from the pilot and repeat experiments

Samples from the pilot experiment (▲) and the repeat experiment (■) are shown with treated (filled) and control (no fill) samples denoted by colour fill. Transplant samples are also shown (⧫)

was the most abundant family with a relative abundance of between 52.9 and 78.1%. Ruminococcaceae was present in all samples but with a lower relative abundance (1.6 to 19.9%). Other families present included Enterobacteriaceae, Enterococcaceae, Peptostreptococcaceae, Clostridiaceae 1 and Erysipelotrichaceae. In contrast, the most abundant family in PT7 samples varied between Ruminococcaceae and Lachnospiraceae. In general, the relative abundance of other families such as Enterobacteriaceae, Enterococcaceae and Bacillaceae was lower in PT7 samples compared to PC7 samples.

In the repeat experiment, two families were prevalent in RC0 samples, Paenibacillaceae and Clostridiaceae 1. While these families were the most abundant in RT0 samples as well, other bacterial families were present. These included Lachnospiraceae, Enterococcaceae, Streptococcaceae, Peptostreptococcaceae and Erysipelotrichaceae. At 3 d.p.h, a more diverse microbiome was present in both treated and control chicks. The composition of RC3 samples was fairly uniform with the most abundant family being Enterobacteriaceae followed by Enterococcaceae and Clostridiaceae 1. Very low levels of Lachnospiraceae were also present (0.1% to 3.4%). With the exception of one sample, where the most abundant family was Enterobacteriaceae, RT3 samples were mainly composed of Lachnospiraceae and Enterococcaceae. As in the pilot experiment, a small relative abundance of Ruminococcaceae was present. Fewer differences in microbiome composition between groups were observed at 7 d.p.h. In both RC7 and RT7 samples, Lachnospiraceae was the most abundant family although RT7 samples tended to have a higher relative abundance. The relative abundance of Ruminococcaceae was similar between the two groups but Enterobacteriaceae had a higher relative abundance in RC7 samples. At 14 d.p.h, the composition of the microbiome was similar between treated and control chicks. Ruminococcaceae and Lachnospiraceae were the most abundant families in the majority of samples. Enterobacteriaceae had a similar relative abundance between RC14 and RT14 samples. Other low abundance taxa present at 14 d.p.h included Lactobacillaceae, Clostridiales vadin BB60 group and Peptostreptococcaceae.

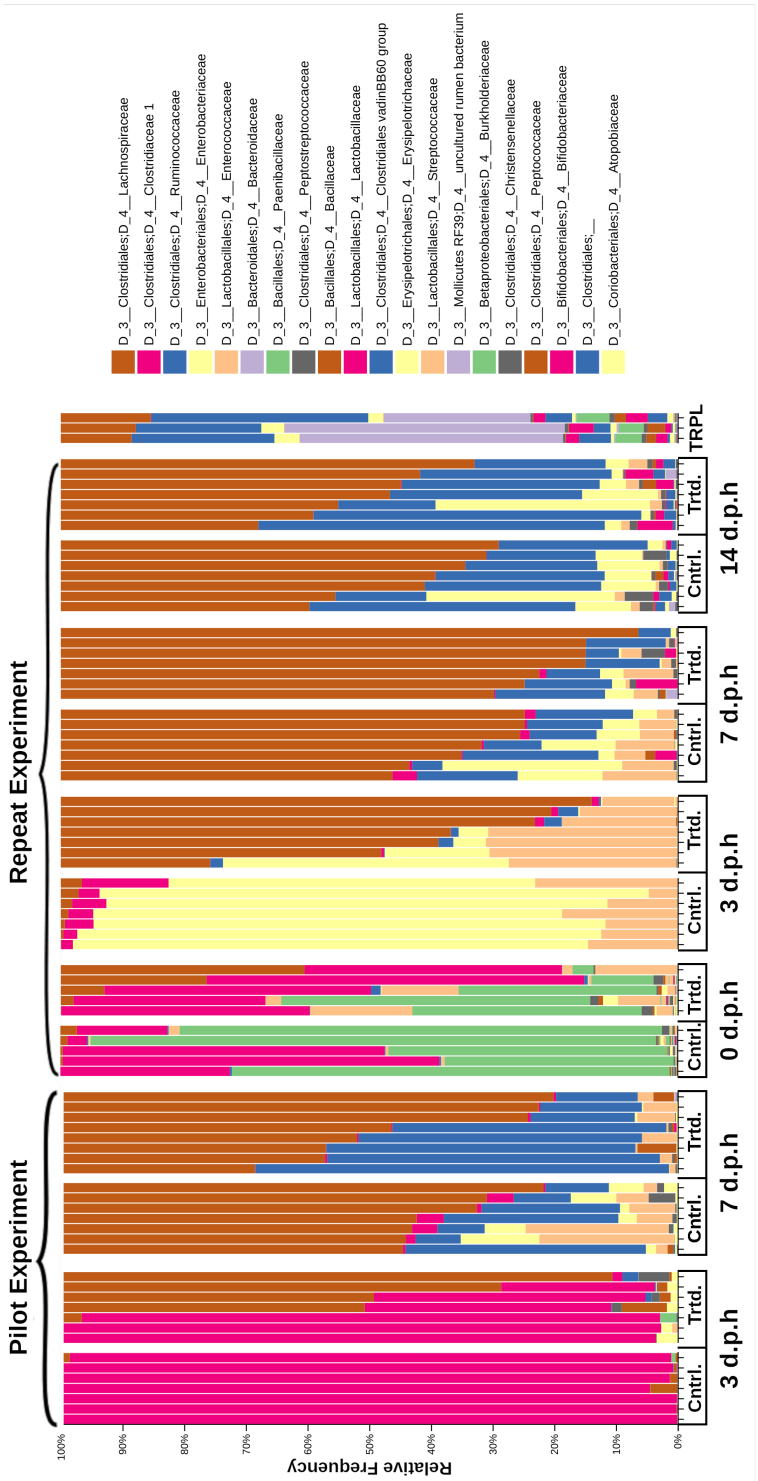


Figure 3.6: Relative abundance of bacterial families in the caecum between 0 and 14 d.p.h in the pilot and repeat experiments Treated (Trtd.); Control (Cntrl.); Transplant Material (TRPL)

Ileum

The taxonomy of the ileal microbiome was observed at the level of genus to differentiate between *Candidatus* Arthromitus and other members of Clostridiaceae (Figure 3.7).

In the pilot experiment, the microbiome of control chicks at 3 d.p.h was formed almost entirely of *Clostridium sensu stricto* 1. While *Clostridium sensu stricto* 1 was the most abundant taxa in treated chicks other genera were present in some samples. Three treated samples had a population of *Enterococus* while others had populations of *Clostridioides*. These differences account for the separation of treated and control samples in the beta diversity PCoA plot (Figure 3.5a). At 7 d.p.h, a more diverse microbiome was present. Control chicks were mainly colonised by *Enterococcus* with a lower relative abundance of *Clostridium sensu stricto* 1. Three samples had a population of *Clostridioides* and all samples had a low relative abundance of *Escherichia-Shigella*. One sample had a high relative abundance of *Candidatus* Arthromitus. In contrast, all except one treated sample had a high relative abundance of *Candidatus* Arthromitus. Other prominent taxa in treated chicks at 7 d.p.h were *Enterococcus* and *Clostridium sensu stricto* 1. *Lactobacillus* was present in one treated sample.

In the repeat experiment, *Clostridium sensu stricto* 1 was the most abundant taxa in both treated and control samples at 0 d.p.h. However, treated samples had a higher relative abundance of *Enterococcus* with a few samples displaying low relative abundance of other taxa such as Lachnospiraceae, *Romboutsia* and *Turicibacter*. At 3 d.p.h, larger differences were visible between treated and control chicks. The dominant genus in control chicks was *Escherichia-Shigella* with *Enterococcus* and *Clostridium sensu stricto* 1 also present in all samples. *Paenibacillus* was present in three samples with *Lactobacillus* present in one control sample. In contrast, treated samples were composed almost entirely of *Enterococcus* with a smaller relative abundance of *Streptococcus* and *Escherichia-Shigella*. These differences at 0 and 3 d.p.h account for the separation of treated and control samples in the beta diversity PCoA plot (Figure 3.5a). At 7 d.p.h, the microbiomes of treated and control chicks was more similar but with some notable differences. *Enterococcus* was the

most abundant genus in both treated and control chicks. Control chicks continued to have a higher relative abundance of *Escherichia-Shigella* and *Clostridium sensu stricto* 1. A more diverse microbiome was present between treated chicks with samples showing varying relative abundance of *Lactobacillus*, *Clostridioides*, *Streptococcus* and Lachnospiraceae. One treated sample had a high relative abundance of *Candidatus* Arthromitus. By 14 d.p.h, the microbiomes of treated and control chicks were similar. The most abundant genus in most samples was *Candidatus* Arthromitus. Other genera present in both groups at 14 d.p.h included *Lactobacillus*, *Enterococcus*, *Romboutsia*, *Escherichia-Shigella* and *Turicibacter*. A small population of Lachnospiraceae and Ruminococcaceae was present in both treated and control samples.

3.3.7 Differentially abundant ASVs between treated and control chicks in the caecum

Pilot Experiment

Gneiss analysis revealed differential ASV abundance between caecal samples from treated and control chicks at 3 and 7 d.p.h. The ASV table was filtered to exclude ASVs with a total frequency of less than 39 reducing the number of ASVs in the analysis from 408 to 306. The overall linear regression model fit was R2 = 0.34 with covariate 'Treatment' accounting for 17.1% of variance. Log ratio balances y0 (β= -19.8, p <0.001), y2 (β= 9.62, p <0.001), y5 (β= -3.97, p = 0.003), y12 (β= -5.42, p <0.001), y14 (β= 6.56, p <0.001) and y27 (β= 7.20, p = 0.006) were significant predictors for the covariate of 'Treatment'. On review of the heatmap, balance y6 was considered to describe ASVs that had a higher log abundance in treated chicks at 3 d.p.h. Figure 3.8 shows the log abundance of ASVs at 3 and 7 d.p.h between treated and control chicks and a summary of balances. Individual log ratios by group for significant balances and balance taxonomy are available in Figure B.3 and Table B.1 respectively. The taxonomy of ASVs identified as differentially abundant between treated and control samples is presented in Table 3.2a with relative abundance of bacterial families in each sample displayed in Figure 3.6. A high number of ASVs assigned

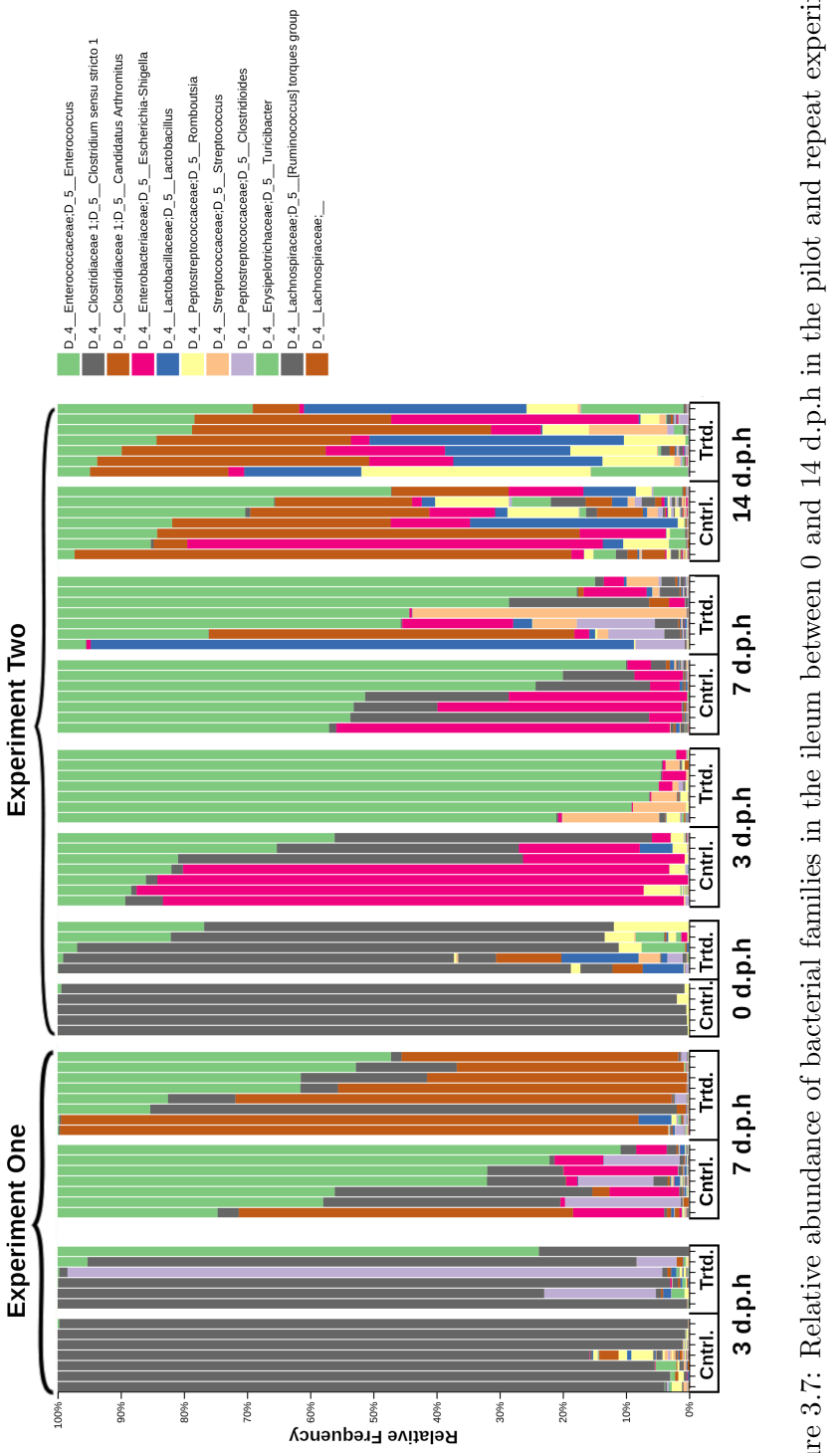


Figure 3.7: Relative abundance of bacterial families in the ileum between 0 and 14 d.p.h in the pilot and repeat experiments Treated (Trtd.); Control (Cntrl.)

to Lachnospiraceae, Bacillaceae, Ruminococcaceae, and Lactobacillaceae were found to have a higher relative abundance in treated samples compared to control samples. Some ASVs were found to have a higher abundance in control compared to treated samples and were assigned to Enterobacteriaceae, Erysipelotrichaceae and Peptostreptococcaceae.

Repeat Experiment

Gneiss analysis revealed differential ASV abundance between caecal samples from treated and control chicks at 0, 3, 7 and 14 d.p.h. The ASV table was filtered to exclude ASVs with a frequency of less than 30 reducing the number of ASVs in the analysis from 633 to 475. The overall linear regression model fit was R2 = 0.31 with covariate 'Treatment' accounting for 9.65% of variance. Log ratio balances y0 (β= 14.2, p <0.001), y5 (β= -6.0, p = 0.001), y10 (β= -5.7, p = 0.009), y14 (β= -7.8, p <0.001), y27 (β= 4.1, p = 0.01) and y28 (β= 2.9, p <0.001) were significant predictors for the covariate 'Treatment'. On review of the heatmap, balance y4 was considered to describe ASVs differentially present in control chicks at 3 d.p.h and balance $y1_{denominator}$ ASVs were considered to be equally abundant between treated and control samples. Balances y5, y14, y27 and y28 contained ASVs already identified as differentially abundant in treated or control samples by other balances. Figure 3.9 shows the log abundance of ASVs at 0, 3, 7 and 14 d.p.h between treated and control chicks along with a summary of balances created by Gneiss analysis. Individual log ratios for significant balances and balance taxonomy are available in Figure B.4 and Table B.2 respectively. The taxonomy of ASVs identified as differentially abundant between treated and control samples is presented in Table 3.2b with relative abundance of bacterial families in each sample displayed in Figure 3.6. A high number of ASVs assigned to Lachnospiraceae, Ruminococcaceae, Clostridiales vadin BB60 group, Bacillaceae, Peptostreptococcaceae and Mollicutes RF39 were found to have a higher relative abundance in treated samples compared to control samples. Some ASVs were found to have a higher abundance in control compared to treated samples and were assigned to Clostridiaceae 1, Enterobacteriaceae and Enterococcaceae.

(a) Pilot Experiment

	Number of ASVs			
Taxonomy	**Total**	**TF**	**CF**	**NF**
Lachnospiraceae	119	80	18	21
Ruminococcaceae	104	46	7	51
Clostridiaceae 1	21	10	10	1
Erysipelotrichaceae	16	4	9	3
Enterobacteriaceae	10	3	3	4
Peptostreptococcaceae	9	4	5	0
Bacillaceae	7	5	0	2
Clostridiales vadinBB60 group	6	2	0	4
Paenibacillaceae	4	3	1	0
Enterococcaceae	3	1	0	2
uncultured rumen bacterium	2	2	0	0
Lactobacillaceae	2	2	0	0
Christensenellaceae	1	0	0	1
Bacillales	1	1	0	0
Microbacteriaceae	1	1	0	0

(b) Repeat Experiment

	Number of ASVs			
Taxonomy	**Total**	**TF**	**CF**	**NF**
Lachnospiraceae	193	90	20	83
Ruminococcaceae	155	79	17	59
Clostridiaceae 1	39	1	5	33
Clostridiales vadinBB60 group	15	7	1	7
Erysipelotrichaceae	12	2	0	10
Peptostreptococcaceae	11	3	0	8
Enterobacteriaceae	8	0	4	4
Enterococcaceae	7	0	3	4
Bacillaceae	7	4	0	3
Lactobacillaceae	5	2	1	2
Christensenellaceae	3	1	0	2
Paenibacillaceae	3	0	0	3
Microbacteriaceae	2	0	0	2
Staphylococcaceae	2	0	0	2
uncultured rumen bacterium	2	2	0	0
Thermaceae	1	0	0	1
Sanguibacteraceae	1	0	0	1
Streptococcaceae	1	1	0	0
Hydrogenophilaceae	1	0	0	1
Burkholderiaceae	1	0	0	1
Propionibacteriaceae	1	0	0	1
Leuconostocaceae	1	0	0	1
Nocardiaceae	1	0	0	1
Peptococcaceae	1	0	0	1
Moraxellaceae	1	0	0	1
Alicyclobacillaceae	1	0	0	1

Table 3.2: A taxonomy summary of ASVs identified as differentially abundant in treated and control caecal samples

More abundant in treated (TF); More abundant in control (CF); Not differentially abundant (NF)

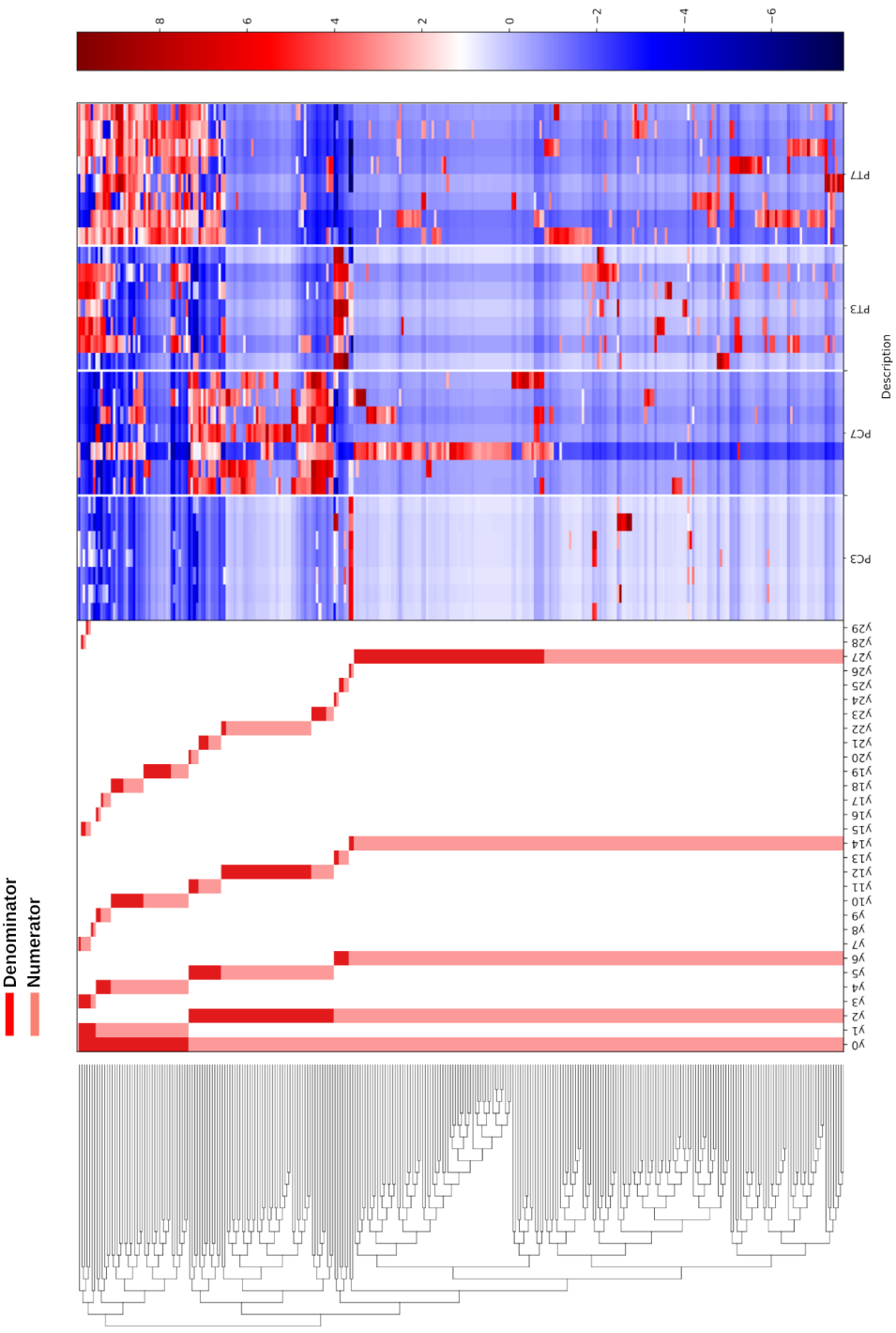


Figure 3.8: ASV log abundance in the caecal microbiome of treated and control chicks in the pilot experiment
Differences in relative abundance between treatments are visible in balances y0, y2, y5, y6, y14 and y27 identified by Gneiss analysis as containing differentially abundant ASVs.

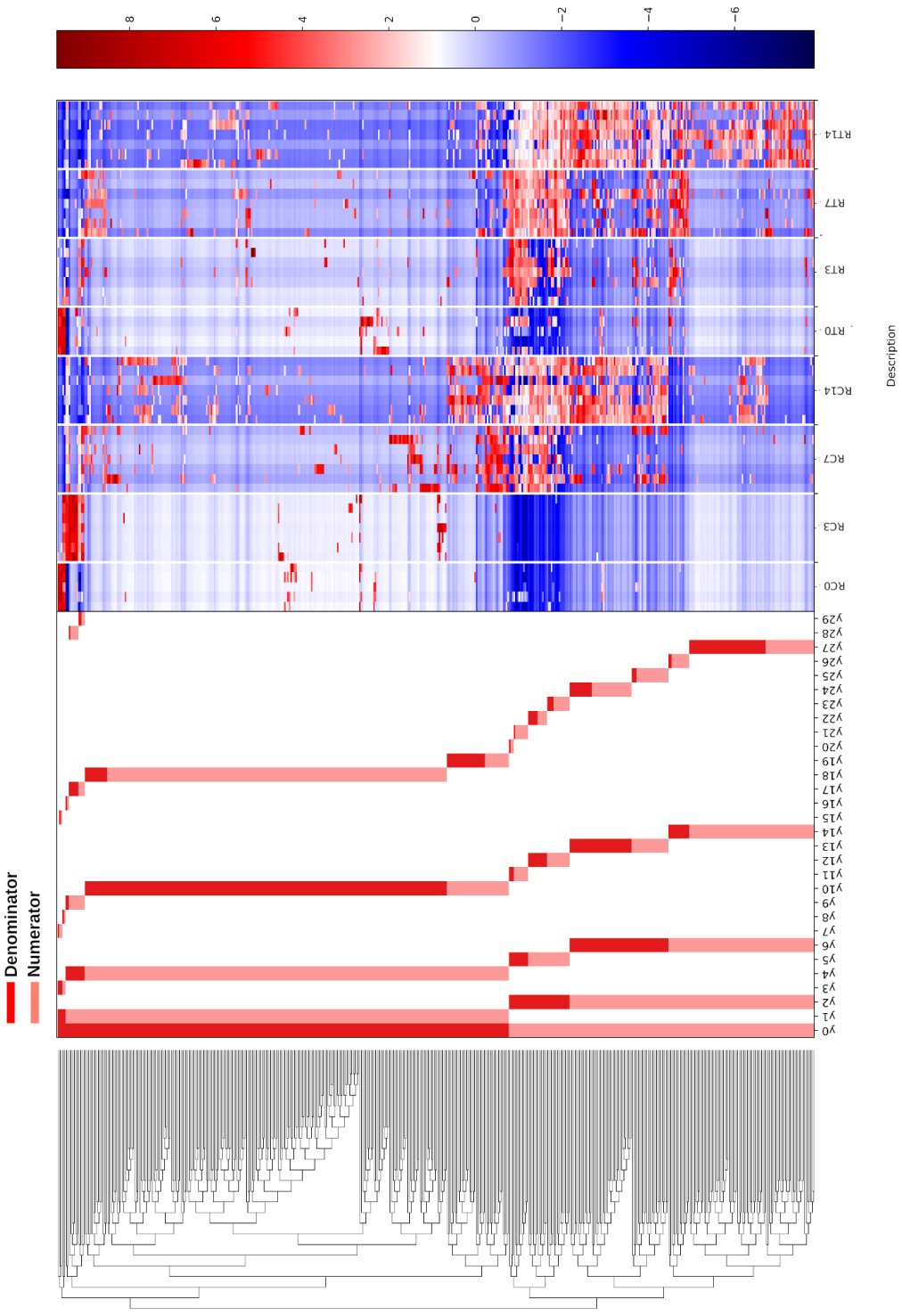


Figure 3.9: ASV log abundance in the caecal microbiome of treated and control chicks in the repeat experiment
Differences in relative abundance between treatments are visible in balances y0, y2, y3, y4, y6, y8, y10 and y14 identified by Gneiss analysis as containing differentially abundant ASVs.

3.3.8 Differentially abundant ASVs between treated and control chicks in the ileum

Pilot Experiment

Gneiss analysis revealed differential ASV abundance between ileal samples from treated and control chicks at 3 and 7 d.p.h. The ASV table was filtered to exclude ASVs with a frequency of less than 18 reducing the number of ASVs in the analysis from 410 to 206. The overall linear regression model fit was R2 = 0.30 with covariate 'Treatment' accounting for 8.2% of variance. No log ratios were significantly different between treatment groups when a correction for multiple tests was applied. In this case, p-values that have not been corrected for multiple tests were used. Log ratio balances y0 (β= -4.6, p = 0.001), y2 (β= -3.5, p = 0.04), y9 (β= -4.2, p = 0.006), y11 (β= -2.8, p = 0.007) and y13 (β= -3.2, p = 0.01) were significant predictors for the covariate of 'Treatment'. Figure 3.10 shows the log abundance of ASVs at 3 and 7 d.p.h between treated and control chicks along with a summary of balances created by Gneiss analysis. Individual log ratios by group for significant balances and balance taxonomy are available in Figure B.5 and Table B.3. The taxonomy of ASVs identified as differentially abundant between treated and control samples is presented in Table 3.3a with relative abundance of bacterial genera in each sample displayed in Figure 3.7. Most ASVs were not differentially abundant between treated and control samples. Of the 28 ASVs assigned to Clostridiaceae 1, 26 were assigned to *Clostridium sensu stricto* 1 and two to *Candidatus* Arthromitus at the genus level. Both *Candidatus* Arthromitus ASVs were identified as more abundant in treated samples.

Repeat Experiment

Gneiss analysis revealed differential ASV abundance between ileal samples from treated and control chicks at 0, 3, 7 and 14 d.p.h. The ASV table was filtered to exclude ASVs with a frequency of less than 28 reducing the number of ASVs in the analysis from 566 to 288. The overall linear regression model fit was R2 = 0.30 with covariate 'Treatment' accounting for 8.1% of variance. Log ratio balances y0 (β= -4.3, p = 0.02), y5 (β= -4.0, p =

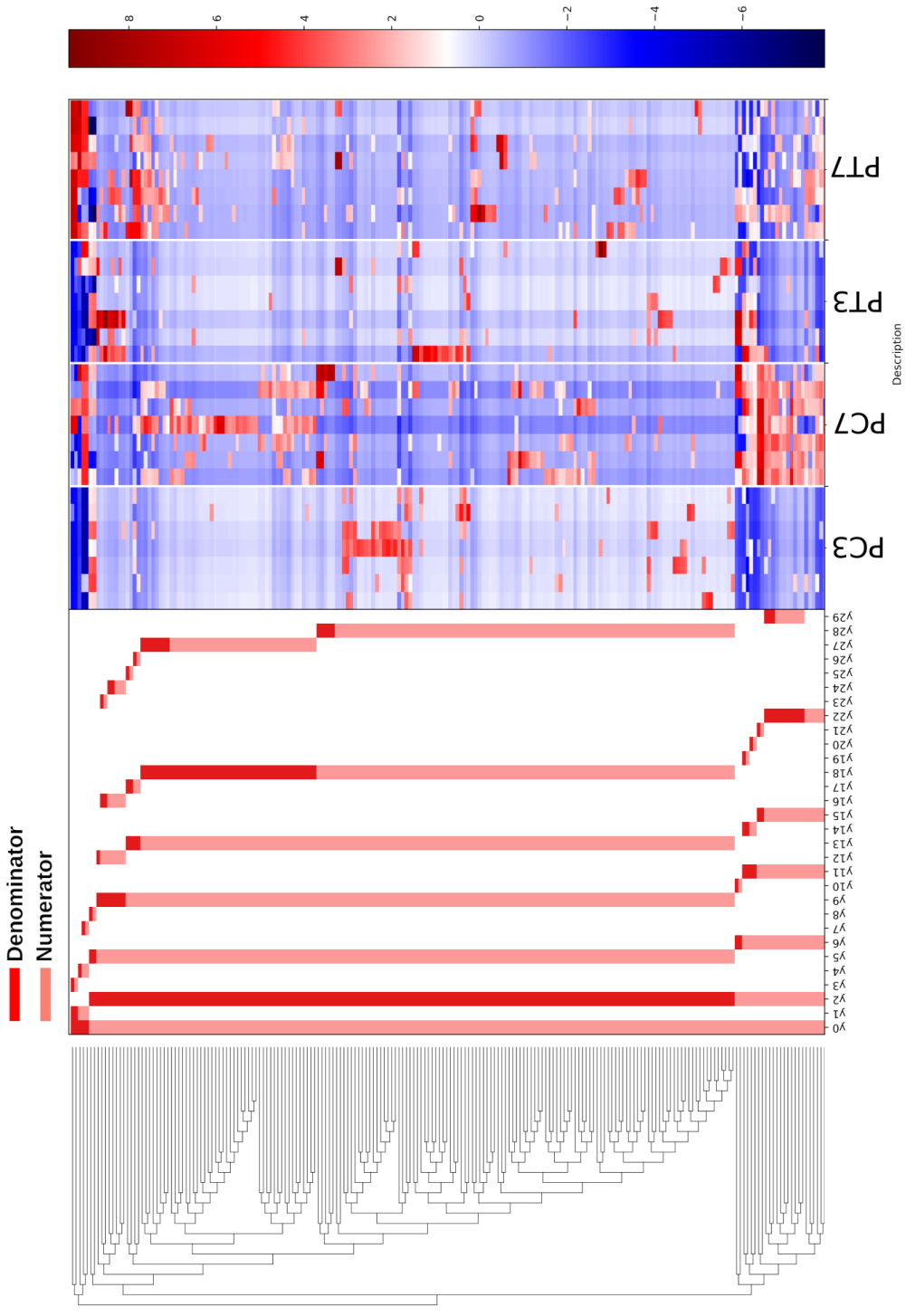


Figure 3.10: ASV log abundance in the ileal microbiome of treated and control chicks in the pilot experiment
Differences in relative abundance between treatments are visible in balances y0, y2, y9, y11 and y13 identified by Gneiss analysis as containing differentially abundant ASVs.

0.002), y11 (β= -3.1, p = 0.001), y21 (β= -6.8, p <0.001) and y28 (β= -5.2, p = 0.001) were significant predictors for the covariate 'Treatment'. On review of the dendrogram heatmap, balance y22 was considered to represent ASVs which had a higher log abundance in treated chicks at 0 and 3 d.p.h and balance y26 was considered to represent ASVs with a higher log abundance in control chicks at 0 d.p.h. Figure 3.11 shows the log abundance of ASVs at 0, 3, 7 and 14 d.p.h between treated and control chicks along with a summary of balances created by Gneiss analysis. Individual log ratios by group for significant balances and balance taxonomy are available in Figure B.6 and Table B.4 respectively. The taxonomy of ASVs identified as differentially abundant between treated and control samples is presented in Table 3.3b with relative abundance of bacterial genera in each sample displayed in Figure 3.7. As with the pilot experiment, most ASVs were not differentially abundant between treated and control samples. A higher proportion of Lachnospiraceae, Ruminococcaceae and Enterobacteriaceae were identified as more abundant in control samples than in the pilot experiment. Equally, a higher proportion of Peptostreptococcaceae were identified as more abundant in treated samples than in the pilot experiment. Of 42 ASVs assigned to Clostridiaceae 1, 39 were assigned to *Clostridium sensu stricto* 1 and three to *Candidatus* Arthromitus. Of the three ASVs assigned to *Candidatus* Arthromitus, two were identified as more abundant in treated samples and one as not differentially abundant between groups.

3.3.9 Successfully transplanted features in the caecum

445 ASVs were defined as present in the transplant material. ASVs present in the transplant material that were not subsequently identified in any samples from the pilot experiment and the repeat experiment were removed from the analysis (n = 274). These were classified as not transplanted. Most of these ASVs were assigned to Ruminococcaceae (n = 125), Clostridiales vadin BB60 group (n = 38), Lachnospiraceae (n = 33), Christensenellaceae (n = 12) and Peptococcaceae (n = 9). ASVs assigned to Bacteroidaceae, Lactobacillaceae, Coriobacteriaceae, Bifidobacteriaceae, Burkholderiaceae and Eggerthellaceae had a high relative abundance in the transplant material (Figure 3.6). However, none of these ASVs were successfully transplanted to the caecum in either the pilot experiment or the repeat

(a) Pilot Experiment

Taxonomy	Number of ASVs			
	Total	TF	CF	NF
Lachnospiraceae	58	5	11	42
Ruminococcaceae	31	2	4	25
Clostridiaceae 1	28	7	1	20
Enterobacteriaceae	14	0	3	11
Peptostreptococcaceae	11	1	0	10
Microbacteriaceae	10	0	0	10
Bacillaceae	9	1	0	8
Lactobacillaceae	8	0	0	8
Erysipelotrichaceae	7	2	0	5
Enterococcaceae	5	3	0	2
Nocardiaceae	3	0	0	3
Staphylococcaceae	3	0	0	3
Pseudomonadaceae	3	0	0	3
Sanguibacteraceae	2	0	0	2
Paenibacillaceae	2	0	0	2
Moraxellaceae	2	0	0	2
Corynebacteriaceae	2	0	0	2
Thermaceae	1	0	0	1
Christensenellaceae	1	0	0	1
Pirellulaceae	1	0	0	1
Alicyclobacillaceae	1	0	0	1
Propionibacteriaceae	1	0	0	1
Burkholderiaceae	1	0	0	1
Micrococcaceae	1	0	0	1
Rhizobiaceae	1	0	0	1

(b) Repeat Experiment

Taxonomy	Number of ASVs			
	Total	TF	CF	NF
Lachnospiraceae	102	31	45	26
Ruminococcaceae	43	5	23	15
Clostridiaceae 1	42	2	8	32
Peptostreptococcaceae	21	8	4	9
Erysipelotrichaceae	11	1	4	6
Enterococcaceae	11	0	3	8
Bacillaceae	10	0	0	10
Enterobacteriaceae	9	0	5	4
Lactobacillaceae	6	2	1	3
Paenibacillaceae	5	0	2	3
Microbacteriaceae	5	0	3	2
Staphylococcaceae	4	0	0	4
Pasteurellaceae	3	0	0	3
Rhizobiaceae	2	0	1	1
Pseudomonadaceae	2	0	0	2
uncultured rumen bacterium	2	1	0	1
Sanguibacteraceae	2	0	1	1
Leuconostocaceae	1	0	0	1
Streptococcaceae	1	1	0	0
Prevotellaceae	1	0	0	1
Planococcaceae	1	0	0	1
Clostridiales vadinBB60 group	1	0	0	1
Christensenellaceae	1	0	1	0
Nocardiaceae	1	0	1	0
Listeriaceae	1	0	0	1

Table 3.3: A taxonomy summary of ASVs identified as differentially abundant in treated and control ileal samples

More abundant in treated (TF); More abundant in control (CF); Not differentially abundant (NF)

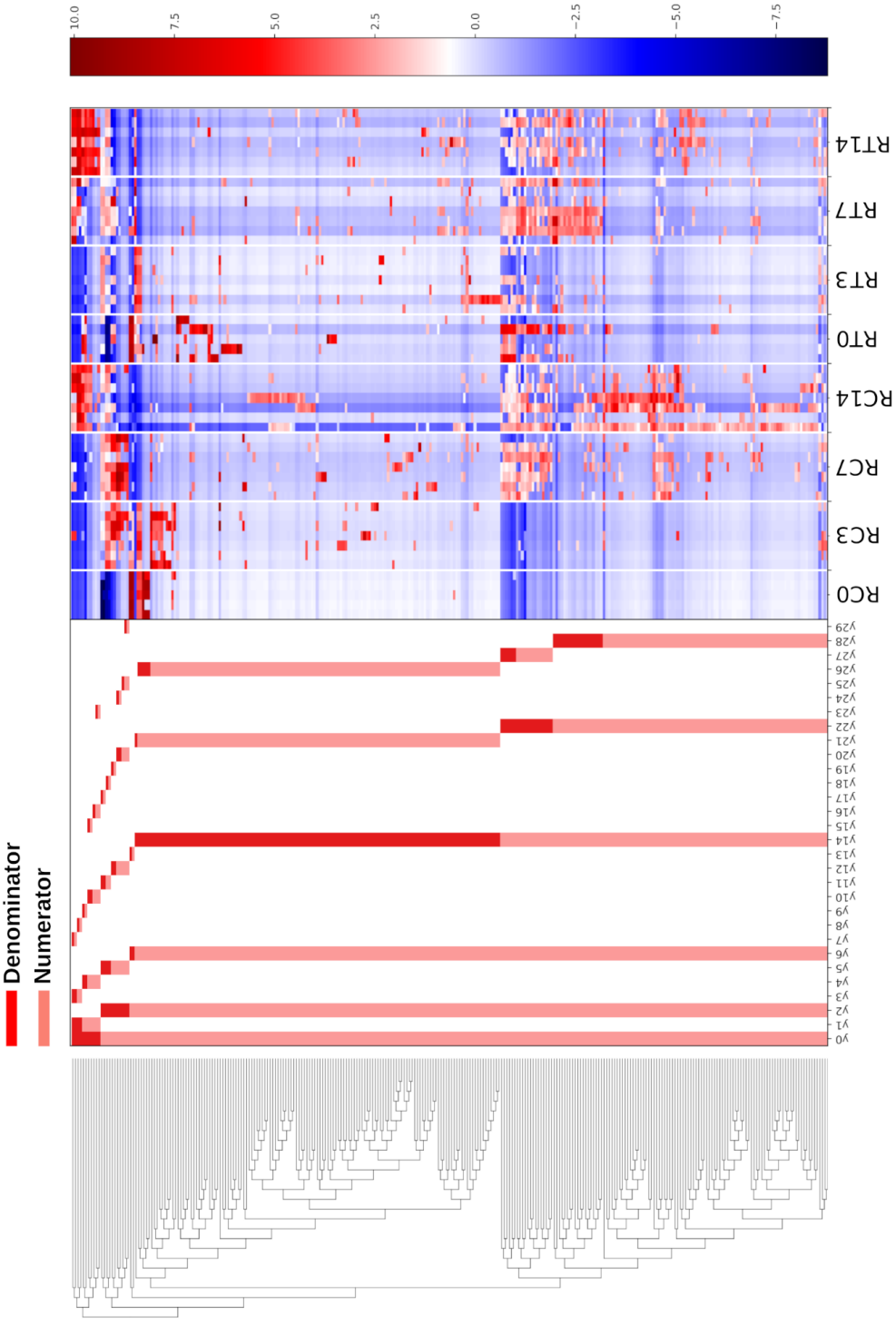


Figure 3.11: ASV log abundance in the ileal microbiome of treated and control chicks in the repeat experiment Differences in relative abundance between treatments are visible in balances y0, y5, y11, y21, y22, y26 and y28 identified by Gneiss analysis as containing differentially abundant ASVs.

experiment.

Pilot Experiment

A total of 56 ASVs, or 12% of ASVs present in the transplant material, were classified as successfully transplanted (Table 3.5a and Figure 3.12a). The taxonomy assignment of successfully transplanted, possibly transplanted and environmental ASVs is shown in Table 3.4a. Only ASVs assigned to Lachnospiraceae, Ruminococcaceae, Erysipelotrichaceae and Mollicutes RF39 (uncultured rumen bacteria) were defined as successfully transplanted. A further 49 ASVs were classified as possibly transplanted of which the majority were assigned to the families Ruminococcaceae and Lachnospiraceae. Other ASVs classified as possibly transplanted were assigned to Clostridiaceae 1, Erysipelotrichaceae, Bacillaceae, Peptostreptococcaceae, Enterobacteriaceae, Enterococcaceae and Christensenellaceae. At the genus level, the two ASVs assigned to Clostridiaceae 1 were identified as *Candidatus* Arthromitus. The remaining 201 ASVs were classified as environmental. Some taxa were almost exclusively classified as environmental including Clostridiaceae 1, Enterobacteriaceae, Peptostreptococcaceae, Bacillaceae, Clostridiales vadin BB60 group, Enterococcaceae, Paenibacillaceae and Lactobacillaceae. 46 ASVs from the transplant which were present in the repeat experiment were not found in samples from the pilot experiment.

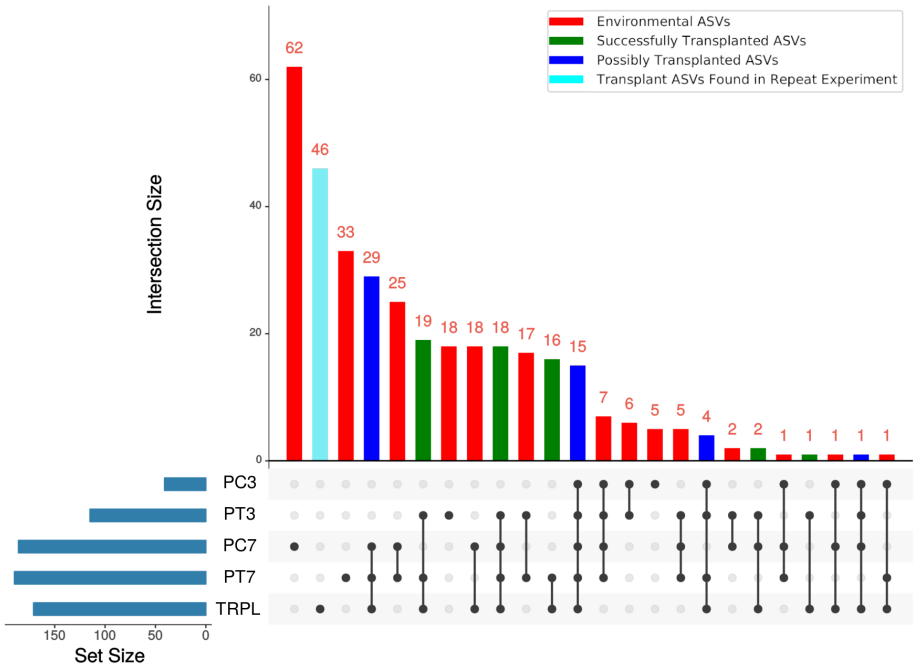
A contingency table (Table 3.5a) shows the overlap between ASVs identified as differentially abundant between treated and control chicks and their classification in terms of transplant success. The relationship between group assignment was significant ($\chi 2$ (4) = 29.2, p <0.001). More ASVs identified as more abundant in control samples were classified as environmental and more ASVs identified as more abundant in treated samples were classified as successfully transplanted than would be expected. Equally, fewer ASVs identified as more abundant in control samples were classified as successfully transplanted and fewer ASVs identified as more abundant in treated samples were classified as environmental than would be expected. A hybrid Sankey diagram was produced to show how the taxonomy of transplanted and environmental ASVs relates to that of ASVs identified as differentially abundant between treated and control samples (Figure 3.13a). ASVs that were identified

as more abundant in treated samples and successfully transplanted were mainly assigned to Lachnospiraceae and Ruminococcaceae with two such ASVs assigned to Erysipelotrichaceae. Nearly all ASVs assigned to Bacillaceae, Clostridiaceae 1, Clostridiales vadin BB60 group, Lactobacillaceae, Enterobacteriaceae, Paenibacillaceae, Peptostreptococcaceae that were more abundant in treated chicks were classified as environmental and therefore could not have been derived from the transplant material. The exceptions to this were one ASV assigned to Bacillaceae and one to Clostridiaceae 1 (assigned to *Candidatus* Arthromitus at the genus level) that were identified as possibly transplanted.

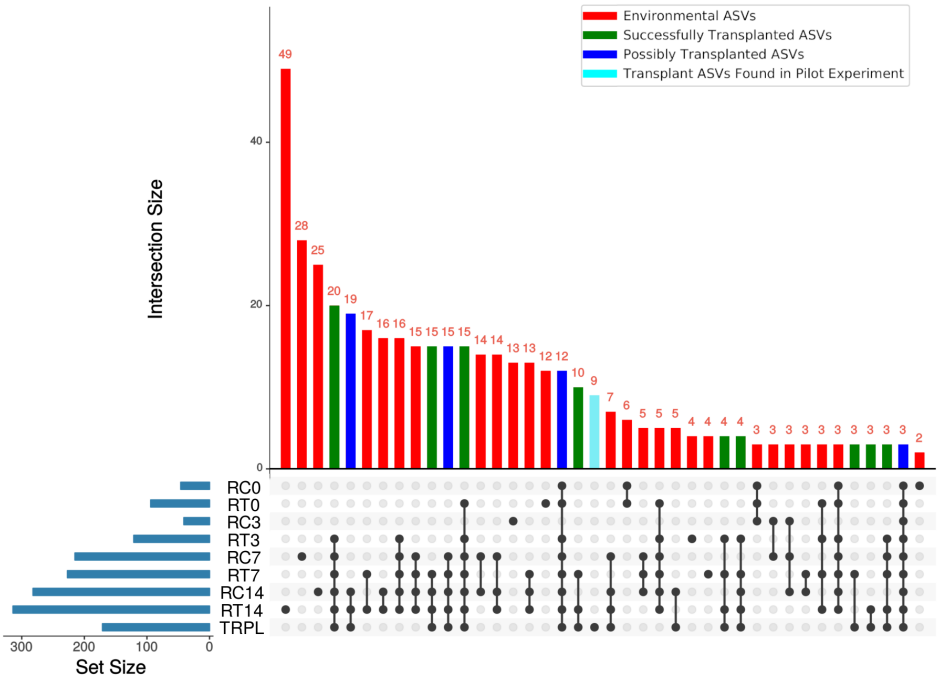
Repeat Experiment

A total of 89 ASVs, or 20% of ASVs present in the transplant material, were classified as successfully transplanted (Table 3.5b and Figure 3.12b). The taxonomy assignment of ASVs is shown in Table 3.4b. The majority were assigned to the families Lachnospiraceae and Ruminococcaceae with other ASVs assigned to Erysipelotrichaceae, Peptostreptococcaceae, Enterobacteriaceae, Clostridiales vadin BB60 group, Bacillaceae and Clostridiaceae 1. The ASV assigned to Clostridiaceae 1 was identified at the genus level as *Candidatus* Arthromitus. A further 37 ASVs were classified as possibly transplanted of which the majority were assigned to the family Ruminococcaceae. Other ASVs classified as possibly transplanted were assigned to Lachnospiraceae, Christensenellaceae, Mollicutes (uncultured rumen bacteria), Clostridiales vadin BB60 group and Clostridiaceae 1 (which was further identified as *Candidatus* Arthromitus). The remaining 349 ASVs were classified as environmental. As for the pilot experiment, some taxa were mainly classified as environmental including Clostridiaceae 1, Clostridiales vadin BB60 group, Erysipelotrichaceae, Peptostreptococcaceae, Enterobacteriaceae, Bacillaceae, Enterococcaceae, Paenibacillaceae and Lactobacillaceae. Nine ASVs from the transplant material which were present in the pilot experiment were not found in samples from the repeat experiment.

A contingency table (Table 3.5b) shows the overlap between ASVs identified as differentially abundant between treated and control chicks and their classification in terms of transplant success. The relationship between group assignment was significant ($\chi 2$ (4) =



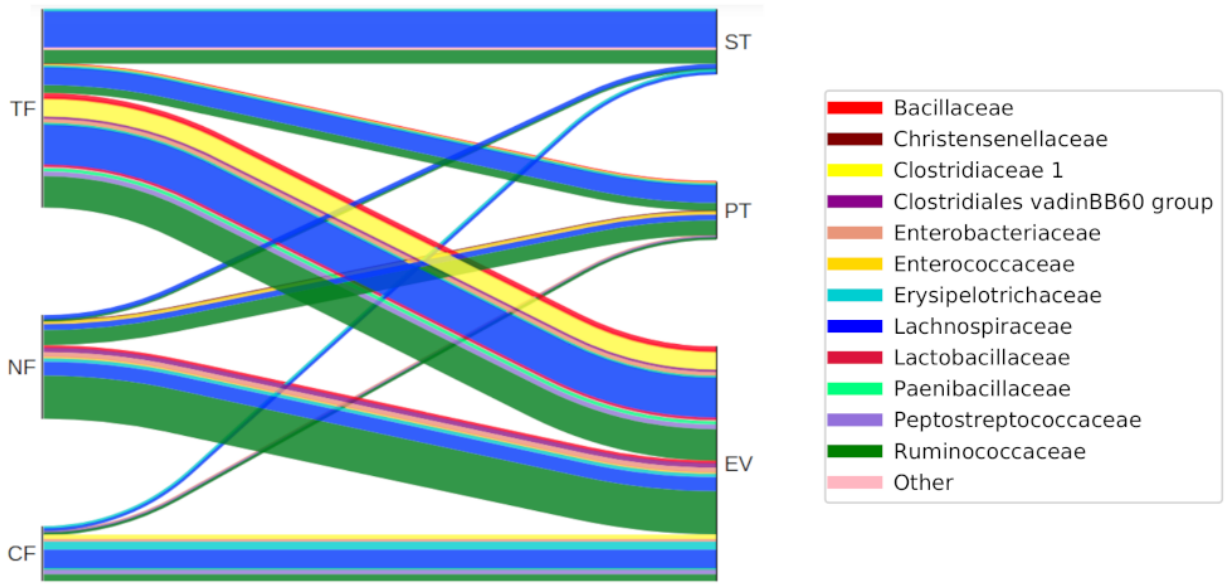
(a) Pilot Experiment



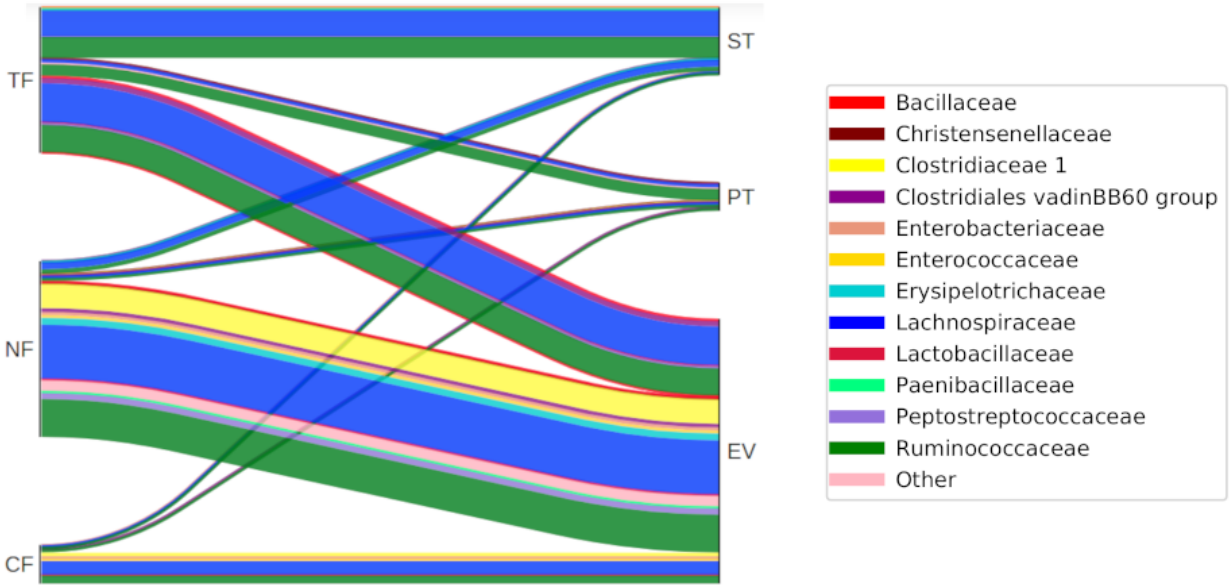
(b) Repeat Experiment

Figure 3.12: ASV intersections between caecal sample groups

Intersections are ordered by size with empty intersections excluded from the visualisation. The bar chart indicates the size of the intersection and is coloured according to ASV classification as environmental, successfully transplanted or possibly transplanted. Intersections are visualised below the bar chart with contributing sample groups identified by black points.



(a) Pilot Experiment



(b) Repeat Experiment

Figure 3.13: The relationship between differentially abundant ASVs and transplant classification in the caecum

Gneiss analysis was used to identify ASVs as more abundant in treated (TF), control (CF) and not differentially abundant (NF). UpSet was used to classify ASVs as successfully transplanted (ST), possibly transplanted (PT) or environmental (EV). In both experiments, the majority of ASVs identified as more abundant in treated samples were also classified as successfully or possibly transplanted leading to the conclusion that differences between treated and control samples can be attributed to transplated ASVs rather than environmental ASVs.

72.8, p <0.001). Results were similar to those from the pilot experiment with more ASVs identified as more abundant in treated samples classified as successfully transplanted and possibly transplanted than would be expected. Fewer ASVs identified as more abundant in control samples were classified as successfully transplanted and fewer ASVs identified as more abundant in treated samples were classified as environmental than would be expected. However, the number of ASVs identified as more abundant in control samples and classified as environmental was close to the expected value. A hybrid Sankey diagram was produced to visualise how the taxonomy of transplanted and environmental ASVs relates to that of ASVs identified as differentially abundant between treated and control samples (Figure 3.13b). The taxonomic profile of ASVs identified as more abundant in treated samples and successfully transplanted was similar to that described for the pilot experiment. These ASVs were mainly assigned to Lachnospiraceae and Ruminococcaceae with two ASVs assigned to Erysipelotrichaceae and one each to Peptostreptococcaceae, Erysipelotrichaceae and Bacillaceae. One ASV assigned to Clostridiaceae 1, identified at the genus level as *Candidatus* Arthromitus, was classified as both more abundant in treated samples and successfully transplanted. Nearly all ASVs assigned to Bacillaceae (the exception being the aforementioned Bacillaceae ASV) and all ASVs assigned to Clostridiales vadin BB60 group, Lactobacillaceae, Streptococcaceae, Peptostreptococcaceae that were more abundant in treated chicks were classified as environmental and therefore could not have been derived from the transplant material.

3.3.10 Successfully transplanted ASVs in the ileum

The same list of ASVs present in the transplant material generated for analysis of caecal transplant success was used to analyse the success of the transplant in the ileum. Three hundred and forty four ASVs from the transplant material were excluded from the analysis as they were not found in any ileal samples from the pilot experiment or the repeat experiment. These were classified as not transplanted. The taxonomic composition of these ASVs was similar to those that were not transplanted to the caecum although there was a higher number of Ruminococcaceae (n = 165), Clostridiales vadin BB60 group (n = 45),

(a) Pilot Experiment

Taxonomy	Number of ASVs			
	Total	**Successfully Transplanted**	**Possibly Transplanted**	**Environmental**
Lachnospiraceae	119	37	20	62
Ruminococcaceae	104	13	21	70
Clostridiaceae 1	21	0	2	19
Erysipelotrichaceae	16	4	1	11
Enterobacteriaceae	10	0	1	9
Peptostreptococcaceae	9	0	1	8
Bacillaceae	7	0	1	6
Clostridiales vadinBB60 group	6	0	0	6
Paenibacillaceae	4	0	0	4
Enterococcaceae	3	0	1	2
Lactobacillaceae	2	0	0	2
uncultured rumen bacterium	2	2	0	0
Bacillales	1	0	0	1
Christensenellaceae	1	0	1	0
Microbacteriaceae	1	0	0	1

(b) Repeat Experiment

Taxonomy	Number of ASVs			
	Total	**Successfully Transplanted**	**Possibly Transplanted**	**Environmental**
Lachnospiraceae	193	45	8	140
Ruminococcaceae	155	36	22	97
Clostridiaceae 1	39	1	1	37
Clostridiales vadinBB60 group	15	1	2	12
Erysipelotrichaceae	12	3	0	9
Peptostreptococcaceae	11	1	0	10
Enterobacteriaceae	8	1	0	7
Bacillaceae	7	1	0	6
Enterococcaceae	7	0	0	7
Lactobacillaceae	5	0	0	5
Christensenellaceae	3	0	2	1
Paenibacillaceae	3	0	0	3
uncultured rumen bacterium	2	0	2	0
Staphylococcaceae	2	0	0	2
Microbacteriaceae	2	0	0	2
Alicyclobacillaceae	1	0	0	1
Propionibacteriaceae	1	0	0	1
Hydrogenophilaceae	1	0	0	1
Thermaceae	1	0	0	1
Peptococcaceae	1	0	0	1
Moraxellaceae	1	0	0	1
Streptococcaceae	1	0	0	1
Leuconostocaceae	1	0	0	1
Burkholderiaceae	1	0	0	1
Nocardiaceae	1	0	0	1
Sanguibacteraceae	1	0	0	1

Table 3.4: The taxonomy of ASVs by transplant success in the caecum

(a) Pilot Experiment

	Higher Abundance in Control	Not Differentially Abundant	Higher Abundance in Treated	Total
Environmental	40 (31)	63 (58)	98 (112)	201
Possibly Transplanted	3 (8)	21 (14)	25 (27)	49
Successfully Transplanted	4 (9)	5 (16)	47 (31)	56
Total	47	89	170	

(b) Repeat Experiment

	Higher Abundance in Control	Not Differentially Abundant	Higher Abundance in Treated	Total
Environmental	41 (37)	206 (171)	102 (141)	349
Possibly Transplanted	5 (4)	9 (18)	23 (15)	37
Successfully Transplanted	5 (10)	17 (43)	67 (36)	89
Total	51	232	192	

Table 3.5: Observed frequencies of ASV classification by differential abundance and transplant success in the caecum

Expected frequencies calculated using a chi squared test of independence are displayed in brackets

Lachnospiraceae (n = 54) and Christensenellaceae (n = 14).

Pilot Experiment

In the pilot experiment, a total of 18 ASVs were classified as successfully transplanted (Table 3.7a and Figure 3.14a). The taxonomy assignment of ASVs is shown in Table 3.6a. ASVs assigned to Lachnospiraceae, Ruminococcaceae, Erysipelotrichaceae, Clostridiaceae 1, Enterobacteriaceae, Peptostreptococcaceae and Bacillaceae were defined as successfully transplanted. The ASV assigned to Clostridiaceae 1 was assigned to *Candidatus* Arthromitus at the genus level. A further 33 ASVs were classified as possibly transplanted of which the majority were assigned to the families Lachnospiraceae and Ruminococcaceae. Other ASVs classified as possibly transplanted were assigned to Erysipelotrichaceae, Lactobacillaceae and Peptostreptococcaceae. The remaining 155 ASVs were classified as environmental. Some taxa were almost exclusively classified as environmental including Clostridiaceae 1, Enterobacteriaceae, Peptostreptococcaceae, Microbacteriaceae, Bacillaceae, Lactobacillaceae, Enterococcaceae and Staphylococcaceae. 31 ASVs from the transplant which were present in the repeat experiment were not found in samples from the pilot experiment.

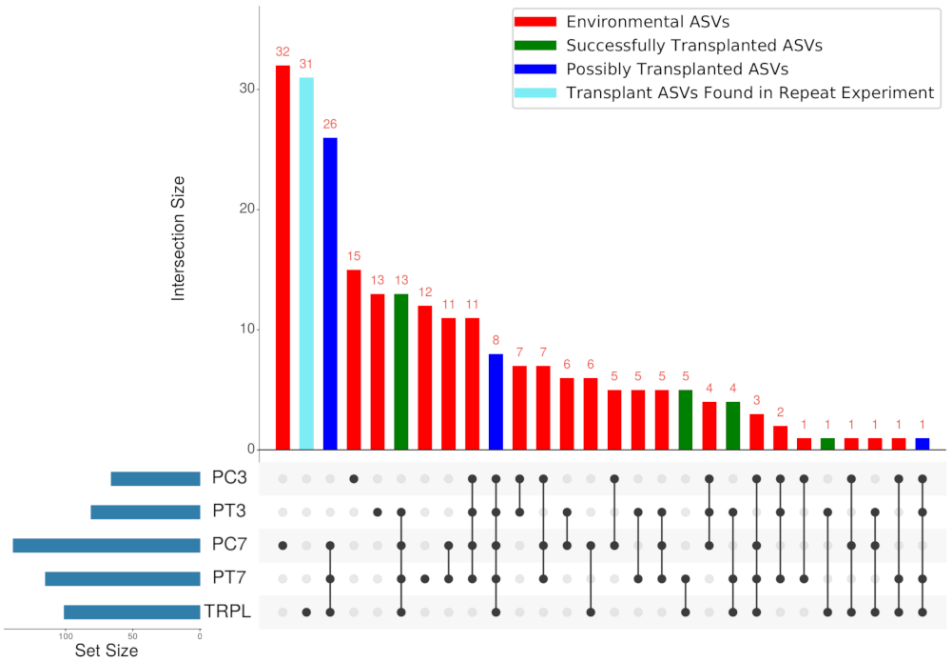
A contingency table (Table 3.7a) showed the overlap between ASVs identified as differentially abundant between treated and control chicks and their classification in terms of transplant success. The relationship between group assignment was significant ($\chi 2$ (4) = 22.8, p <0.001). Actual values were close to expected values for ASVs identified as environmental or possibly transplanted and those more abundant in control samples, not differentially abundant or more abundant in treated samples. Slightly more ASVs identified as more abundant in treated samples were classified as successfully transplanted than would be expected. A hybrid Sankey diagram was produced to visualise how the taxonomy of transplanted and environmental ASVs related to that of ASVs identified as differentially abundant between treated and control samples (Figure 3.15a). Four ASVs identified as both more abundant in treated samples and successfully transplanted were assigned to Lachnospiraceae with the remaining three assigned to Bacillaceae, Erysipelotrichaceae (assigned to the genus *Turicibacter*) and Clostridiaceae 1 (assigned to genus *Candidatus*

Arthromitus). Only one ASV identified as more abundant in treated samples was classified as possibly transplanted and was assigned to Erysipelotrichaceae. All ASVs assigned to Clostridiaceae 1 (with the exception of the aforementioned ASV), Peptostreptococcaceae, Enterococcaceae and Ruminococcaceae identified as more abundant in treated samples were classified as environmental and therefore could not have been derived from the transplant material.

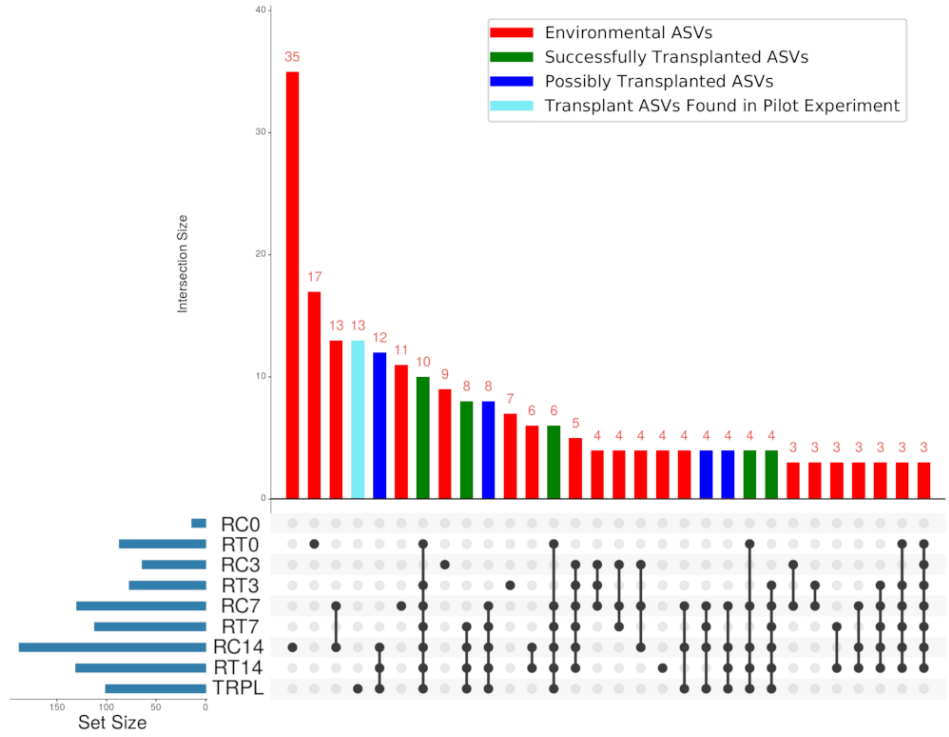
Repeat Experiment

In the repeat experiment, a total of 50 ASVs were classified as successfully transplanted (Table Table 3.7b and Figure 3.14b). The taxonomy assignment of ASVs is shown in Table 3.6b. The majority were assigned to the families Lachnospiraceae and Ruminococcaceae with other ASVs assigned to Clostridiaceae 1, Erysipelotrichaceae, Peptostreptococcaceae, Enterobacteriaceae, and Enterococcaceae. Of the three ASVs assigned to Clostridiaceae 1, two were identified at the genus level as *Candidatus* Arthromitus and one as *Clostridium sensu stricto* 1. A further 29 ASVs were classified as possibly transplanted of which the majority were assigned to the family Lachnospiraceae. Other ASVs classified as possibly transplanted were assigned to Ruminococcaceae, Christensenellaceae, Mollicutes (uncultured rumen bacteria), Enterobacteriaceae and Enterococcaceae. The remaining 209 ASVs were classified as environmental. As for the pilot experiment, some taxa were mainly classified as environmental including Clostridiaceae 1, Peptostreptococcaceae, Bacillaceae, Enterococcaceae, Erysipelotrichaceae, Enterobacteriaceae, Lactobacillaceae, Paenibacillaceae, Microbacteriaceae and Staphylococcaceae. 13 ASVs from the transplant which were present in the pilot experiment were not found in samples from the repeat experiment.

A contingency table (Table 3.7b) showed the overlap between ASVs identified as differentially abundant between treated and control chicks and their classification in terms of transplant success in the repeat experiment. The relationship between group assignment was significant ($\chi 2$ (4) = 84.8, p <0.001). Actual values were close to expected values for ASVs identified as more abundant in control samples and environmental, and more



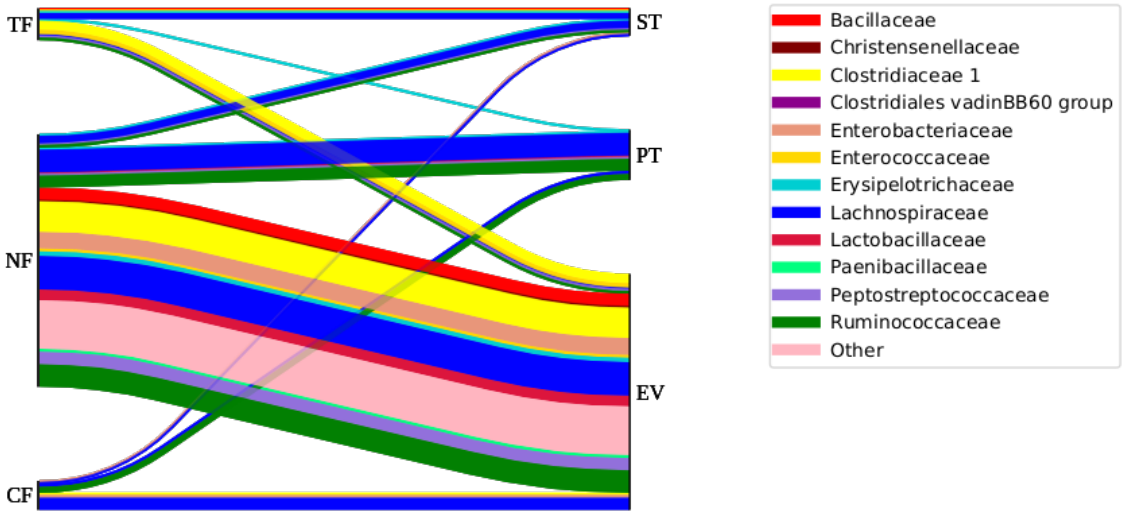
(a) Pilot Experiment



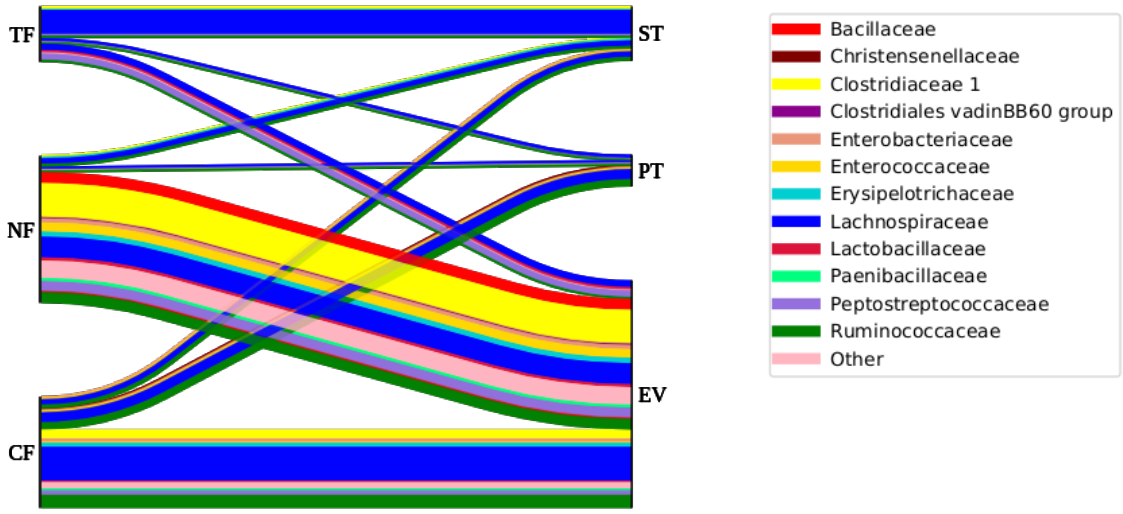
(b) Repeat Experiment

Figure 3.14: ASV intersections between ileal sample groups.

Intersections are ordered by size with empty intersections excluded from the visualisation. The bar chart indicates the size of the intersection and is coloured according to ASV classification as environmental, successfully transplanted or possibly transplanted. Intersections are visualised below the bar chart with contributing sample groups identified by black points



(a) Pilot Experiment



(b) Repeat Experiment

Figure 3.15: The relationship between differentially abundant ASVs and transplant classification in the ileum

Gneiss analysis was used to identify ASVs as differential abundant in treated (TF), control (CF) and not differentially abundant (NF). UpSet was used to classify ASVs as successfully transplanted (ST), possibly transplanted (PT) or environmental (EV). Unlike in the caecum, most ASVs were identified as NF and EV. A small number of ASVs in both experiments were identified as more abundant in treated chicks and successfully transplanted.

abundant in treated samples and possibly transplanted. Fewer ASVs identified as more abundant in treated samples were classified as environmental and fewer ASVs identified as more abundant in control samples were classified as successfully transplanted than would be expected. More ASVs identified as more abundant in treated samples were classified as successfully transplanted than would be expected. Unexpectedly, more ASVs identified as more abundant in control samples were classified as possibly transplanted than would be expected suggesting that a greater proportion of possibly transplanted ASVs were of environmental than transplant origin. A hybrid Sankey diagram was produced to visualise how the taxonomy of transplanted and environmental ASVs relates to that of ASVs identified as differentially abundant between treated and control samples (Figure 3.15b). Of the ASVs identified as both more abundant in treated samples and successfully transplanted most were assigned to Lachnospiraceae. Two ASVs each were assigned to Peptostreptococcaceae (at the genus level one was assigned to *Romboutsia* and the other to *Clostridoides*), Ruminococcaceae and Clostridiaceae 1 (both were assigned to *Candidatus* Arthromitus at the genus level) with one ASV assigned to Erysipelotrichaceae (assigned to the genus *Turicibacter*). Most Peptostreptococcaceae and all Lactobacillaceae ASVs that were identified as more abundant in treated samples were classified as environmental and therefore could not have been derived from the transplant material.

3.3.11 Quantitative PCR

Caecum

Genera within Lachnospiraceae have generally been placed in *Clostridium* cluster XIVa while genera within Ruminococcaceae have generally been placed in *Clostridium* cluster IV (Lopetuso *et al.*, 2013; Onrust *et al.*, 2015). As such, primers for *Clostridium* cluster XIVa&b were used to estimate the abundance of Lachnospiraceae and primers for *Clostridium* cluster IV were used to estimate the abundance of Ruminococcaceae.

In the repeat experiment, treated chicks had significantly more *Clostridium* cluster XIVa&b at 0 d.p.h ($t = 22.14$, $p < 0.001$) as no DNA from this primer was detected in

(a) Pilot Experiment

Taxonomy	Number of ASVs			
	Total	**Successfully Transplanted**	**Possibly Transplanted**	**Environmental**
Lachnospiraceae	58	10	17	31
Ruminococcaceae	31	2	12	17
Clostridiaceae 1	28	1	0	27
Enterobacteriaceae	14	1	0	13
Peptostreptococcaceae	11	1	1	9
Microbacteriaceae	10	0	0	10
Bacillaceae	9	1	0	8
Lactobacillaceae	8	0	1	7
Erysipelotrichaceae	7	2	2	3
Enterococcaceae	5	0	0	5
Staphylococcaceae	3	0	0	3
Pseudomonadaceae	3	0	0	3
Nocardiaceae	3	0	0	3
Moraxellaceae	2	0	0	2
Sanguibacteraceae	2	0	0	2
Corynebacteriaceae	2	0	0	2
Paenibacillaceae	2	0	0	2
Burkholderiaceae	1	0	0	1
Pirellulaceae	1	0	0	1
Micrococcaceae	1	0	0	1
Rhizobiaceae	1	0	0	1
Alicyclobacillaceae	1	0	0	1
Christensenellaceae	1	0	0	1
Thermaceae	1	0	0	1
Propionibacteriaceae	1	0	0	1

(b) Repeat Experiment

Taxonomy	Number of ASVs			
	Total	**Successfully Transplanted**	**Possibly Transplanted**	**Environmental**
Lachnospiraceae	102	32	14	56
Ruminococcaceae	43	9	10	24
Clostridiaceae 1	42	3	0	39
Peptostreptococcaceae	21	2	0	19
Erysipelotrichaceae	11	2	0	9
Enterococcaceae	11	1	1	9
Bacillaceae	10	0	0	10
Enterobacteriaceae	9	1	1	7
Lactobacillaceae	6	0	0	6
Microbacteriaceae	5	0	0	5
Paenibacillaceae	5	0	0	5
Staphylococcaceae	4	0	0	4
Pasteurellaceae	3	0	0	3
uncultured rumen bacterium	2	0	2	0
Sanguibacteraceae	2	0	0	2
Rhizobiaceae	2	0	0	2
Pseudomonadaceae	2	0	0	2
Listeriaceae	1	0	0	1
Nocardiaceae	1	0	0	1
Prevotellaceae	1	0	0	1
Clostridiales vadinBB60 group	1	0	0	1
Planococcaceae	1	0	0	1
Christensenellaceae	1	0	1	0
Leuconostocaceae	1	0	0	1
Streptococcaceae	1	0	0	1

Table 3.6: The taxonomy of ASVs by transplant success in the ileum

(a) Pilot Experiment

	Higher Abundance in Control	Not Differentially Abundant	Higher Abundance in Treated	Total
Environmental	11 (14)	131 (125)	13 (16)	155
Possibly Transplanted	6 (3)	26 (27)	1 (3)	33
Successfully Transplanted	2 (2)	9 (14)	7 (2)	18
Total	19	166	21	

(b) Repeat Experiment

	Higher Abundance in Control	Not Differentially Abundant	Higher Abundance in Treated	Total
Environmental	72 (74)	120 (98)	17 (37)	209
Possibly Transplanted	19 (10)	5 (14)	5 (5)	29
Successfully Transplanted	11 (18)	10 (23)	29 (9)	50
Total	102	135	51	

Table 3.7: Observed frequencies of ASV classification by differential abundance and transplant success in the ileum

Expected frequencies calculated using a chi squared test of independence are displayed in brackets

control samples from 0 d.p.h (Figure 3.16a). In both experiments treated chicks had significantly more *Clostridium* cluster XIVa&b at 3 d.p.h (Pilot Experiment: $t = 7.24$, $p < 0.001$; Repeat Experiment: $t = 11.3$, $p < 0.001$). The difference between treated and control chicks continued to be significant in the repeat experiment at 7 d.p.h ($t = 4.0$, $p < 0.001$) but not in the pilot experiment. There was no significant difference in *Clostridium* cluster XIVa&b abundance between treated and control chicks at 14 d.p.h.

At 0 d.p.h there was no significant difference in *Clostridium* cluster IV abundance between control and treated samples, as this taxa was only detected in one treated sample (Figure 3.16b). At 3 d.p.h, significantly more *Clostridium* cluster IV were detected in treated chicks than control chicks from the repeat experiment ($t = 4.9$, $p < 0.001$) but the result was not quite significant in the pilot experiment ($t = 1.95$, $p = 0.07$). By 7 and 14 d.p.h there was no significant difference in *Clostridium* cluster IV abundance between treated and control chicks.

The initial pattern of colonisation of treated chicks is also notable. *Clostridium* cluster XIVa&b was detected with a high abundance and in all treated samples at 0 d.p.h while the abundance of *Clostridium* cluster IV was relatively low in comparison. There was a high range of intra-group variation of *Clostridium* cluster IV and XIVa&b abundance at 0 d.p.h in treated chicks. This supports the earlier observation that the transplant was not uniformly successful across all treated chicks. There were also differences in the abundance of *Clostridium* cluster IV between treated chicks from the pilot experiment and the repeat experiment. Treated chicks from the repeat experiment had a higher abundance of *Clostridium* cluster IV at 3 d.p.h when compared to those from the pilot experiment ($t = 3.63$, $p = 0.002$). This supports the earlier observation that the transplant was more successful in the repeat experiment than the pilot experiment in terms of transfer of these taxa.

No Enterobacteriaceae were detected in samples from either group at 0 d.p.h in the repeat experiment (Figure 3.16c). In the repeat experiment at 3 d.p.h there was significantly less Enterobacteriaceae in treated chicks ($t = -5.42$, $p < 0.001$). However, in the pilot experiment, the opposite result was obtained with significantly higher levels of Enter-

obacteriaceae detected in treated chicks (t = 3.54, p = 0.005). At 7 d.p.h, significantly less Enterobacteriaceae was detected in treated chicks in both experiments (Pilot Experiment: t = -5.24, p <0.001 ; Repeat Experiment: t = -2.85, p = 0.01). On average, the abundance of Enterobacteriaceae was lower in treated chicks at 14 d.p.h but the difference was not quite significant (t = -1.95, p = 0.07). There was a large inter-experiment variation in Enterobacteriaceae abundance with higher abundance detected at 3 d.p.h in the repeat experiment.

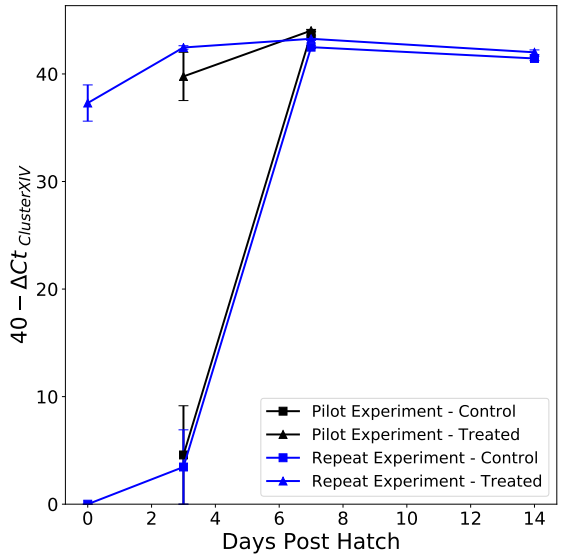
High levels of *Clostridium* were detected in both groups at 0 d.p.h in the repeat experiment with no significant difference between treated and control chicks (Figure 3.16d). Significantly less *Clostridium* was detected in treated samples at 3 d.p.h in the repeat experiment (t = -7.78, p <0.001) but significantly more *Clostridium* was detected in treated samples at 7 d.p.h (t = 3.5, p = 0.002). There were no significant differences in *Clostridium* abundance between treated and control chicks in the pilot experiment at 7 d.p.h or at 14 d.p.h in the repeat experiment.

Ileum

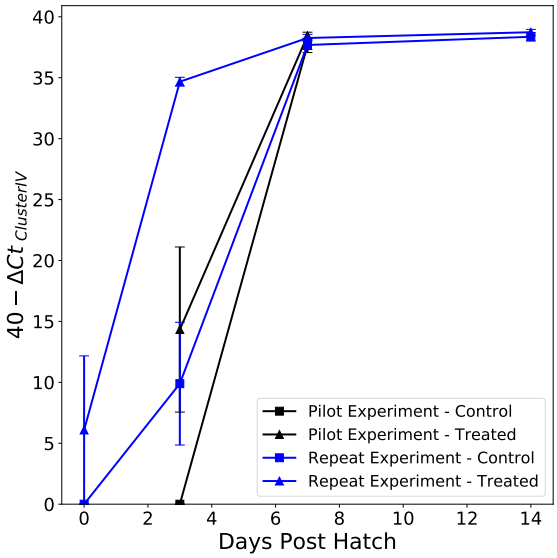
No Enterobacteriaceae were detected in either group at 0 d.p.h in the repeat experiment (Figure 3.17a). There was significantly less Enterobacteriaceae in treated chicks compared to control chicks at 3 d.p.h in the repeat experiment (t = -4.78, p <0.001) and 7 d.p.h in the pilot experiment (t = -9.27, p <0.001). There was no significant difference between treated and control chicks at other time points.

There was no significant difference in *Clostridium* abundance between treated and control chicks at any time point except at 3 d.p.h in the repeat experiment (Figure 3.17b) where the abundance was lower in treated chicks (t = -4.33, p <0.001).

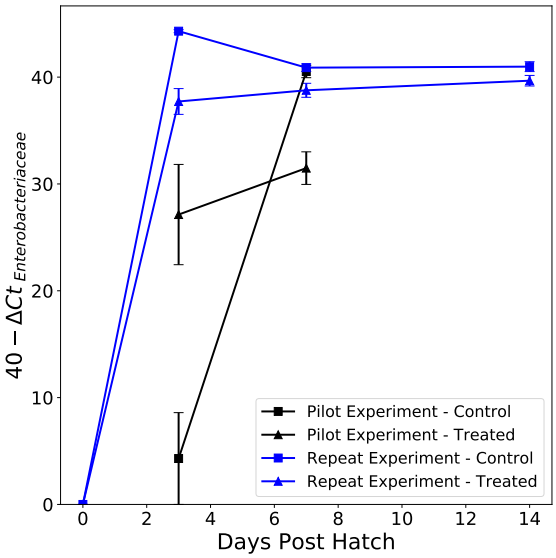
Candidatus Arthromituswas not present in the ileum until 7 d.p.h (Figure 3.17c). At 7 d.p.h, the abundance of *Candidatus* Arthromituswas significantly higher in treated chicks in the pilot and repeat experiments (t = 4.35, p <0.001 and t = 2.97, p = 0.008 respectively). The average abundance of *Candidatus* Arthromituswas higher in treated chicks at 14 d.p.h but the difference was not significant (t = 1.88, p = 0.08).



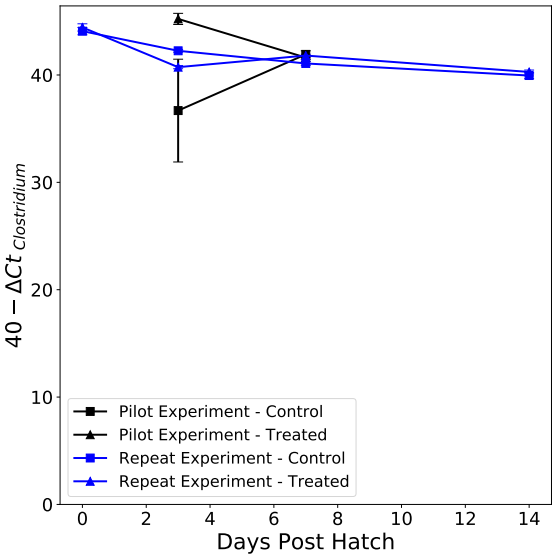
(a) *Clostridium* cluster XIVa&b



(b) *Clostridium* cluster IV

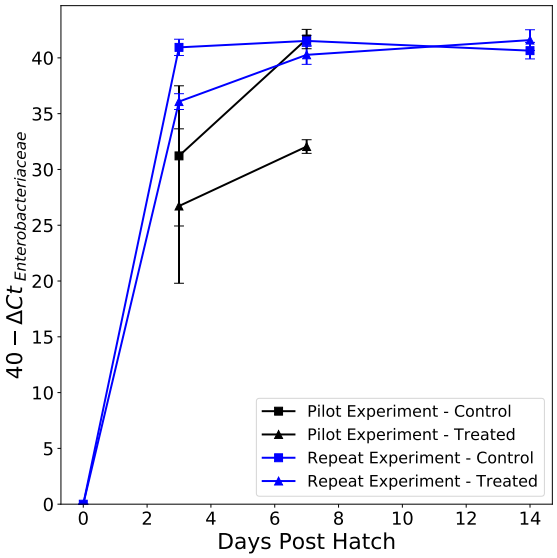


(c) Enterobacteriaceae

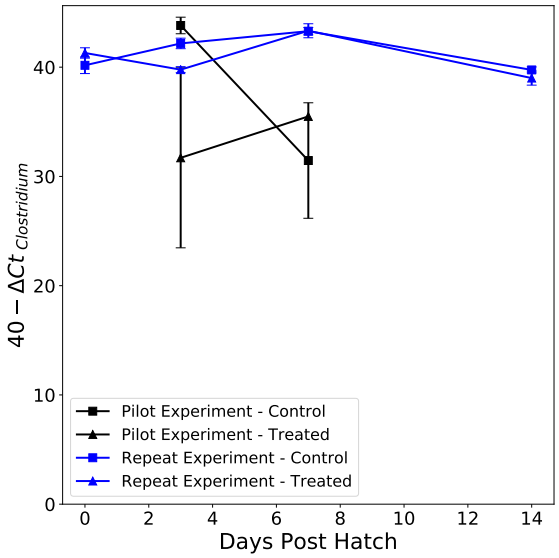


(d) *Clostridium*

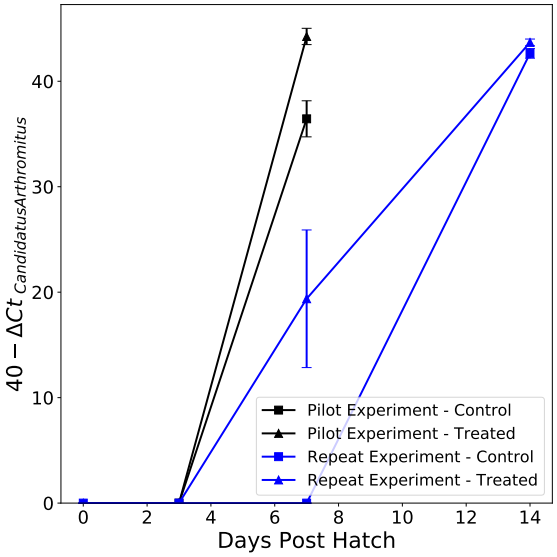
Figure 3.16: Relative abundance of bacterial taxa in the caecum determined by quantitative PCR



(a) Enterobacteriaceae



(b) *Clostridium*



(c) *Candidatus* Arthromitus

Figure 3.17: Relative abundance of bacterial taxa in the ileum determined by quantitative PCR

3.3.12 Histology

Histological examination of ileal and caecal tonsil tissues from the repeat experiment at 0, 3, 7 and 14 d.p.h was conducted to observe morphological parameters associated with intestinal development such as villus height and width, epithelial cell height and crypt mitotic figure counts.

Significantly more mitotic figures (Figures 3.18a and 3.18b) were recorded in the crypts of control chicks at 3 d.p.h in the ileum ($t = 3.53$, $p = 0.008$) and caecal tonsil ($t = 2.81$, $p = 0.03$). There was no significant difference in mitotic figure counts between age groups in the ileum ($F = 3.1$, $p = 0.09$) and caecal tonsil ($F = 0.18$, $p = 0.94$).

There were no significant differences in epithelial cell height, villus height or villus width between treated and control chicks at any age (Figures 3.18c, 3.18d and 3.18e). There was a statistically significant difference in villus height ($F = 41.6$, $p < 0.001$), villus width ($F = 18.1$, $p < 0.001$) and epithelial cell height ($F = 58.9$, $p < 0.001$) between age groups. *Post hoc* comparisons revealed that villus height was significantly different between 0 and 3 d.p.h ($p = 0.001$), 3 and 7 d.p.h ($p = 0.001$) and 7 and 14 d.p.h ($p = 0.001$). Villus width was significantly different between 0 and 3 d.p.h ($p = 0.01$) and 7 and 14 d.p.h ($p = 0.001$). Epithelial cell height was significantly different between 0 and 3 d.p.h ($p = 0.001$) and 3 and 7 d.p.h ($p = 0.001$).

SFB were observed in the ileum from 7 d.p.h with presence on ileal histology correlating with presence of *Canidadatus* Arthromitus detected by qPCR. In the repeat experiment, SFB were also seen in the caecal tonsil in close proximity to to epithelial cells and in the lumen (Figure 3.19a).

No bacteria were found in the caecal crypts of any chicks at 0 d.p.h or control chicks at 3 d.p.h, however, bacteria were identified in the caecal crypts of four treated chicks (Figure 3.19b). At 7 d.p.h, four treated and six control chicks were positive for caecal crypt bacteria. At 14 d.p.h, only occasional bacteria were observed in the caecal crypts of treated or control chicks.

3.3.13 Immunohistochemistry

Tissue from chicks at 3 d.p.h was examined as this was the time point when most differences were found between the microbiota of treated and control chicks. No significant differences in counts of CD4, CD8α, CD8β, γδ TCR and Bu1 cells were found between treated and control caecal tonsils at 3 d.p.h.

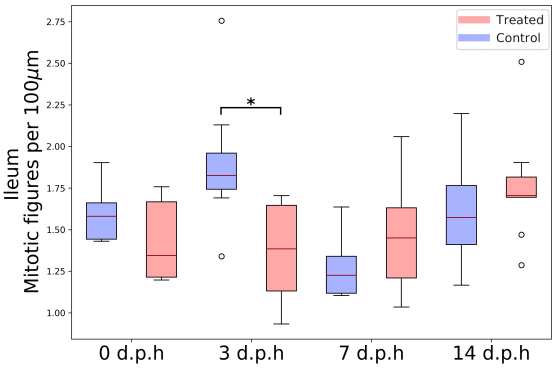
3.4 Discussion

3.4.1 The embryonic gut is sterile until hatch

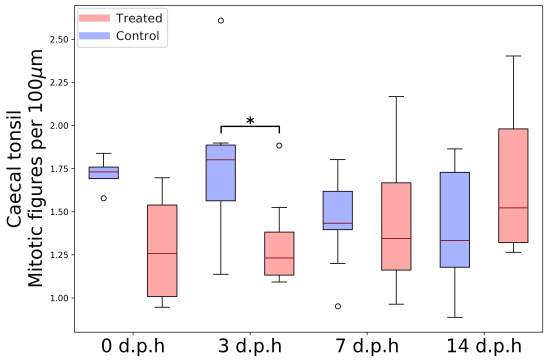
This study demonstrated that there were no detectable bacteria present in the embryonic gut at 18 d.i. It could be argued that detection of PCR amplicons using gel electrophoresis was not sensitive enough to detect small numbers of bacteria within the embryonic gut. However, it's reasonable to suppose that a bacterial population too small to be detected using PCR would also be too small to have an impact in the face of overwhelming colonisation by other bacterial taxa at hatch. It is likely that the chicken gut remains sterile until hatch when bacteria present on the egg surface are the first to colonise.

3.4.2 Topical application of caecal contents accelerated microbiome development

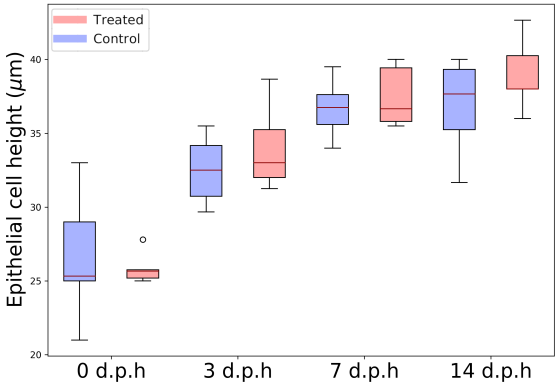
Inoculation of the egg surface with diluted adult caecal contents was sufficient to transplant spore-forming bacteria to chicks with the result of accelerating caecal microbiome development. In treatment and control groups microbial succession followed the same pattern across both experiments. The microbiome of day-old chicks was poorly diverse and composed of environmental bacteria, in this case Clostridiaceae 1 and Paenibacillaceae. This pattern of a low diversity, environmental microbiome is well established in the current literature and has been observed many times (Donaldson *et al.*, 2017; Pedroso *et al.*, 2005; Ballou *et al.*, 2016) as well as being consistent with the observations made in Chapter 2. The order of succession whereby environmental bacteria were replaced first by Lachnospiraceae



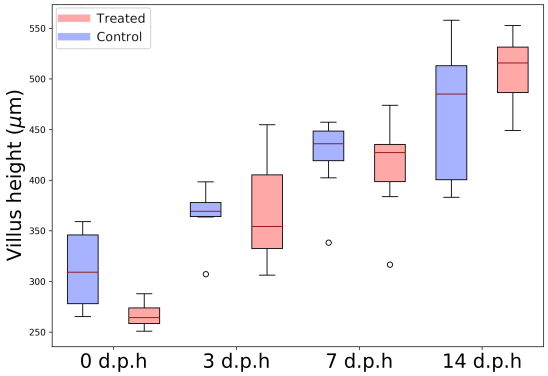
(a) Mitotic counts in ileal crypts



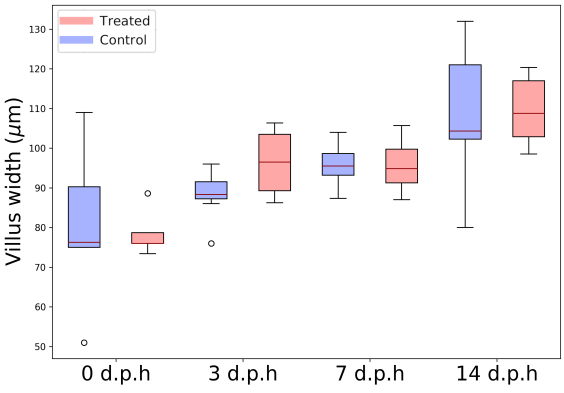
(b) Mitotic counts in caecal tonsil crypts



(c) Mean epithelial cell height



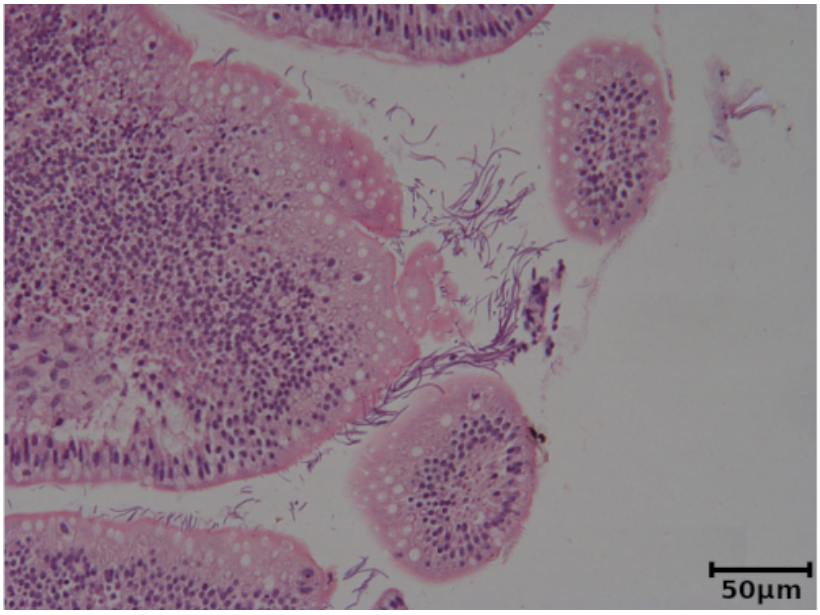
(d) Mean villus height



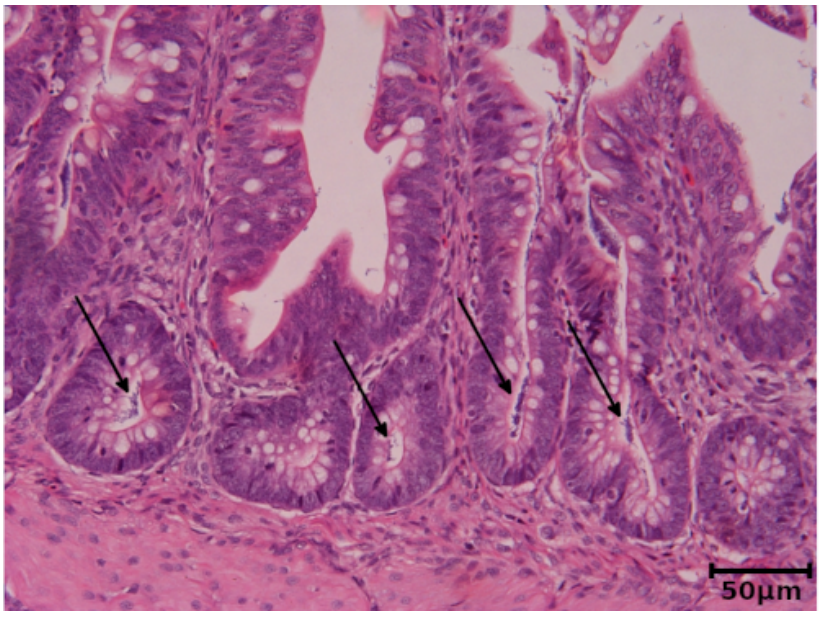
(e) Mean villus width

Figure 3.18: Histological measurements taken from ileal and caecal tonsil tissue

Significant differences between treated and control chicks from the same time point are indicated (*)



(a) Segmented filamentous bacteria in close approximation to the caecal tonsil epithelium at 14 d.p.h. Scale bar = 50µm, magnification: x200



(b) Presence of bacteria in the caecal tonsil crypts at 7 d.p.h. Scale bar = 50µm, magnification: x200

Figure 3.19: Photographs of histological features from the caecal tonsil

and then Ruminococcaceae and other Clostridiales was common across treated and control chicks which was similar to the order of succession described in Chapter 2. However, the speed of succession was faster in treated chicks in both experiments with an initial strong colonisation by Lachnospiraceae followed by an increase in Ruminococcaceae.

Many Ruminococcaceae ASVs were classified as successfully or possibly transplanted suggesting that these ASVs were present at 0 and 3 d.p.h but were unable to colonise the caecum initially. This suggests that alterations to caecal conditions by Lachnospiraceae or some other unknown host factor are a prerequisite for colonisation by Ruminococcaceae.

The presence of ASVs common to the transplant material from as early as a few hours post hatch shows that elements of the caecal microbiota can be successfully transplanted to chicks by topical application to the egg surface. Most of the successfully transplanted ASVs were assigned to Lachnospiraceae and Ruminococcaceae which differs from previously published results. Pedroso *et al.* (2016) found only one OTU assigned to Lachnospiraceae was transferred to treated chicks after inoculating eggs with an *in ovo* injection of a commercial CE product. In contrast, we found that the majority of transferred features were assigned to Lachnospiraceae, Ruminococcaceae and other Clostridiales. However, topical application of caecal contents was unable to transplant several important taxa such as Bacteroidaceae, Lactobacillaceae and Bifidobacteriaceae. A recent study of whole genome sequences from caecal bacteria revealed that genes enabling sporulation were found within most Gram-positive Firmicutes, such as Lachnospiraceae and Ruminococcaceae, with the exception of Lactobacillaceae (Medvecky *et al.*, 2018). No Bacteroidetes isolates were spore-forming, however, 73% of Bacteroidetes isolates were microaerotolerant and able to survive air exposures of 24 hours (Medvecky *et al.*, 2018). Similarly, the bifidobacteria isolated from the chicken gut are non-spore forming (Petr and Rada, 2001). This difference in environmental survival strategy explains the pattern of transplanted features observed in this study. Spores would be able to survive on the egg surface and colonise the chick at hatch whereas non-spore forming members of the caecal microbiota would not survive the 72 hours from the last treatment to hatch. Alternatively, since bacterial viability was not assessed in the transplant material, the storage and handling of the caecal content may

have negatively impacted the survival of taxa that were not transplanted. The inability of a topical egg treatment of diluted adult caecal content at 18 days of incubation to transfer Bacteroidaceae, Lactobacillaceae and Bifidobacteriaceae exposes a major weakness of the technique whether that is due to oxygen exposure during treatment or reduced viability due to storage. Bacteroidaceae is considered a core member of the chicken caecal microbiota (Johnson *et al.*, 2018; Oakley *et al.*, 2014*b*; Wei *et al.*, 2013) while members of Bifidobacteriaceae have been positively correlated with increased bird weight (Johnson *et al.*, 2018). Any future experiments aiming to transplant an adult caecal microbiota would need to take these taxa into account by delivering treatments immediately after hatch, either directly to the chick or into the environment. Exploring different methods of bacterial preservation by using more appropriate storage media to improve bacterial viability provides another avenue for future investigation. This is supported by recent evidence from an experiment in which the development of the caecal microbiome was compared between chicks exposed to a hen for 24 hours and unexposed control chicks. Bacteroidaceae and Actinobacteriaceae were successfully transferred from the hen to chicks (Kubasova *et al.*, 2019). The authors concluded that Firmicutes such as Lachnospiraceae and Ruminococcaceae were not transferred which contradicts the findings presented above. However, this conclusion was based on a lower relative abundance of these taxa in hen-exposed compared to unexposed chicks (Kubasova *et al.*, 2019). As previously discussed, the increased abundance of Bacteroidaceae in hen-exposed chicks would have reduced the relative abundance of Lachnospiraceae and Ruminococcaceae. Since no quantitative PCR was conducted to confirm a reduced absolute abundance such a conclusion cannot be made.

Accelerated microbiota succession was more evident in the caecum but some taxa associated with a more mature intestinal microbiome were present earlier in ileal samples from treated chicks. This included ASVs assigned to *Romboutsia* and *Turicibacter*. Both of these genera were present in the ilea of treated chicks at 0 d.p.h, however, the taxaplot shows that they were not able to persist in the ileum and were not present again until 14 d.p.h when they colonised both treated and control chicks. As with succession in the caecum where Lachnospiraceae appeared to colonise before Ruminococcaceae, it's possible

that conditions in the neonatal ileum were not conducive to colonisation by taxa more associated with a mature microbiota. Another taxon identified as successfully transplanted was *Candidatus* Arthromitus. In both experiments, this taxon had a higher abundance at earlier time points in treated chicks. The abundance of *Candidatus* Arthromituswas studied in more detail in the ileum due to its importance as an immunostimulatory bacteria (Ivanov *et al.*, 2009; Klaasen *et al.*, 1993; Umesaki *et al.*, 1999). Consistent with previous experiments, *Candidatus* Arthromituswas absent in the ileum until 7 d.p.h (Liao *et al.*, 2012). At 7 d.p.h, the abundance of *Candidatus* Arthromituswas significantly higher in treated chicks in both experiments although colonisation by this taxon appears to have occurred earlier in the pilot experiment in both treated and control chicks. The environmental factors influencing *Candidatus* Arthromituscolonisation have not been explored although increased abundance was noted in the ilea of chicks housed on reused litter (Cressman *et al.*, 2010).The transplant material may have contained *Candidatus* Arthromitus spores that transferred to treated chicks resulting in a higher abundance once ileal conditions were suitable for colonisation. Alternatively, the presence of transplanted bacteria in treated chicks may have created favourable metabolic or immunological conditions allowing earlier and greater colonisation by *Candidatus* Arthromitus. Treatments which result in early colonisation by *Candidatus* Arthromitus should be of interest to poultry producers as earlier colonisation by this taxon has been positively correlated with body weight (Johnson *et al.*, 2018). In this study, SFB were found in the caecal tonsil on histology. The presence of SFB in close approximation to the caecal tonsil epithelium was previously reported in 1978 (Glick *et al.*, 1978). While previous studies have been conducted to investigate the role of SFB on immune development in the ileum of mice (Ivanov *et al.*, 2009; Umesaki *et al.*, 1999), no studies focus on similar effects in either the ileum or caecal tonsil of chicken.

3.4.3 Transplant success differed between experiments

The transplant was more successful in the repeat experiment as evidenced by improved early transplant uptake and persistence of significant differences in alpha diversity until 7 d.p.h. The reason for this variability is hard to assess over two experiments and with

a small number of chicks. Since the storage and application of transplant material was uniform across both experiments, uncontrolled variables such as the initial microbiome or other environmental bacteria may have affected transplant success. For example, the initial microbiome in the pilot experiment was formed of Clostridiaceae 1 while that of the repeat experiment was a mixture of Clostridiaceae 1 and Paenibacillaceae. It's possible that Clostridiaceae 1 were better able to resist and prevent colonisation by Lachnospiraceae than Paenibacillaceae.

3.4.4 Transplant success differed between organs

The transplant was more successful in the caecum than the ileum. This is evidenced by more ASVs identified as more abundant in treated chicks in the caecum as well as a higher number of ASVs classified as successfully and possibly transplanted. Additionally, in ileal samples from the repeat experiment, more ASVs identified as having a higher abundance in control samples were also classified as possibly transplanted suggesting that possibly transplanted ASVs were more likely to be derived from the environment than the transplant. This absence of ileal colonisation by bacteria from the transplant material is not surprising given the evidence presented in Chapter 2 that the ileal microbiome is distinct from that of the caecum. However, some ileum-specific taxa were successfully transplanted including ASVs assigned to *Candidatus* Arthromitus, *Romboutsia* and *Turicibacter*, although as previously discussed it appears that the latter two were unable to persist in the ileum. It's likely that caecal content contains a low abundance of ileal microbiota which are flushed there from the small intestine. As such, a lack of organ specific bacteria in the transplant material is an unsatisfactory explanation for the lack of transplant success in the ileum. As in the caecum, a taxon's ability to form spores will have influenced transplant success. In Chapter 2, the ileum was colonised by Lactobacillaceae and Enterococcaceae at early time points. Neither of these taxa are spore-formers and would have been unable to survive on the egg-shell surface (Fisher and Phillips, 2009). However, those taxa which were identified as successfully transplanted, albeit transiently, such as *Candidatus* Arthromitus and *Romboutsia*, are capable of forming spores which would have persisted on the eggshell

until hatch (Gerritsen *et al.*, 2017).

3.4.5 Transplantation did not accelerate intestinal development

This study did not find statistically significant differences in intestinal morphology between treated and control chicks, except that the mitotic figure count was higher in both the ileum and the caecum of control chicks at 3 d.p.h. The caecal microbiota of treated and control chicks at this time point was markedly different with control chicks mainly colonised by Enterobacteriaceae. If mitotic figure count is reflective of epithelial cell replacement rates, this could imply that the presence of Enterobacteriaceae increased epithelial cell replacement. Equally, it could be argued that the lack of Lachnospiraceae and Ruminococcaceae may have induced higher epithelial cell turnover in control chicks since the bacterial metabolite butyrate decreases apoptosis of normal enterocytes (Guilloteau *et al.*, 2010). Body weight was also significantly different between treated and control chicks at 3 d.p.h. As with ileal epithelial turnover, if this difference were attributable to the microbiota it is not possible to distinguish if the cause was a negative effect of Enterobacteriaceae or positive effect of Lachnospiraceae and Ruminococcaceae.

No significant differences in immune cell populations were found between treated and control chicks at 3 d.p.h despite large differences in caecal microbiota. While these differences did not have an impact on the number of immune cells in the caecal tonsil, it remains possible that the presence of different bacterial species stimulates differential gene expression in immune cells since a role of SCFA producing bacteria in immune development has been studied in other species (Corrêa-Oliveira *et al.*, 2016; Meijer *et al.*, 2010).

The presence of bacteria in caecal tonsil crypts has not previously been reported in chickens. This observation was age dependent with sparse bacteria observed at 14 d.p.h compared to 3 and 7 d.p.h. This raises the prospect that the presence of bacteria in the caecal tonsil crypts has some role in immune development which subsequently excludes them from this niche. It was not possible to determine the taxonomy of these bacteria, however, its likely that they were Lachnospiraceae or Ruminococcaceae due to the absence of bacteria in the caecal tonsil crypts of all chicks at 0 d.p.h and control chicks at 3 d.p.h.

Additionally, it was demonstrated in Chapter 2 that these taxa have a higher relative abundance in caecal mucus compared to lumen contents.

3.4.6 Practical applications of topical caecal transplants

A possible use for caecal microbiome transplants in chicks is the competitive exclusion of potential pathogens such as Enterobacteriaceae and *Clostridium* during the first week post-hatch. In this regard, these experiments produced mixed results. In the repeat experiment there was a noticeable difference in the colonisation of Enterobacteriaceae with treated chicks showing a consistently lower abundance. In contrast, treated chicks had a higher abundance of Enterobacteriaceae at 3 d.p.h than control chicks in the pilot experiment. It should be noted that the diluted adult caecal content contained Enterobacteriaceae which could have been transplanted. The role of some Enterobacteriaceae in the chicken caecal microbiota is unclear. While *Escherichia coli* has the potential for pathogenicity, it is often found in the caeca of healthy chickens. As such, the higher abundance of Enterobacteriaceae in control chicks may not be a cause for concern. However, large blooms of Enterobacteriaceae unopposed by other taxa, such as that in control chicks from the repeat experiment, are unlikely to be beneficial to the host. In this regard, the transplant was successful as a similar overgrowth of Enterobacteriaceae was avoided in treated chicks. The lower abundance of Enterobacteriaceae in treated chicks was likely due to the presence of SCFA producing bacteria such as Lachnospiraceae and Ruminococcaceae. Previous studies have found an inhibitory effect of SCFAs on Enterobacteriaceae growth both *in vitro* and *in vivo* (Van Der Wielen *et al.*, 2000). The treatment had less of an impact on the abundance of *Clostridium*. The most significant species of *Clostridium* in terms of chicken health is *Clostridium perfringens* which has been linked to necrotic enteritis in chicks. Direct challenges using this species and other more significant pathogens such as *Campylobacter* and *Salmonella* are required to further explore how caecal microbiota transplants can affect pathogen abundance in the caecum.

Commercial CEPs are often marketed as suitable early treatments for chicks that benefit microbiome development and pathogen exclusion. Pedroso *et al.* (2016) inoculated eggs

with such a product by injecting 0.1ml of CPCE into the amniotic fluid at 18 d.i. Some OTUs present in the probiotic were able to persist in treated chicks up to 7 d.p.h. However, Pedroso *et al.* (2016) concluded that only two OTUs were able to persist from 0 to 32 d.p.h. Additionally, the taxonomy of OTUs identified as transferred from the probiotic to chicks differed to those in the study described above. Pedroso *et al.* (2016) described a range of bacterial taxa among the transferred OTUs, however, only one belonged to the family Lachnospiraceae. In contrast, it was found that the majority of transferred features were assigned to Lachnospiraceae, Ruminococcaceae and other Clostridiales. These bacterial taxa are poorly characterised as many examples have yet to be cultured and fully described. Commercial CEPs are collections of culturable bacteria, but the results of this study show that the ASVs most likely to successfully colonise and persist within the chicken caecum belonged to taxa that are challenging to culture in the laboratory such as Lachnsopiraceae, Ruminococcaceae and, to a lesser degree, Clostridiales vadin BB60 and Mollicutes RF39. It's unlikely that current commercial CEPs have an optimal bacterial composition for long term colonisation of treated chicks. Development of CEPs should focus on including the aforementioned bacterial taxa as these have been shown to readily colonise newly hatched chicks and persist within the caecum.

Donaldson *et al.* (2017) treated eggs one day before hatching with adult caecal contents from either high or low FCR birds. As in the present study, the caecal content used was rich in Bacteroides that was not transferred to the recipient birds. However, they did not observe significant differences in alpha diversity or the pattern of bacterial colonisation between treated and control birds, although treatment had a significant influence on beta diversity measured by weighted and unweighted UniFrac indices. Additionally, they found that the treatment was insufficient to transfer the donor's FCR profile to the recipients or significantly reduce bird-to-bird microbiota variation. In contrast, the treatment presented in this chapter resulted in increased alpha diversity and accelerated succession. One possible explanation for this disparity is the delivery method used. Donaldson *et al.* (2017) describe swabbing the egg surface with diluted adult caecal content. It's possible that this resulted in lower numbers of bacterial spores or vegetative cells which were unable to colonise the

recipient chicks. It could be concluded that a successful topical transplant of the caecal microbiome requires a larger dose of diluted adult caecal content. Eggs in the experiments described above would have received a total of 0.6ml of diluted caecal content across three treatments. Additionally, multiple treatments may have allowed for an accumulation of viable bacterial spores more akin to the effect of close contact with the hen during incubation.

3.5 Conclusion

In summary, three topical applications of dilute adult caecal content to the eggshell was sufficient to transplant elements of the caecal microbiota to newly hatched chicks resulting in accelerated development of the caecal microbiota. However, while important members of the caecal microbiota such as Lachnospiraceae and Ruminococcaceae were successfully transplanted, topical application failed to transplant Bacteroidaceae or Lactobacillaceae. Topical application of characterised bacterial communities to the eggshell during incubation provides a mechanism to transfer a desirable intetestinal microbiota to chicks and reduce colonisation by possible pathogens. However, treatment ending at 18 d.i only successfully transferred spore-forming bacteria with further experiments required to determine whether non spore-forming microbiota can be transplanted by topical treatments to eggs or chicks in the hours before or after hatch. The treatment did not result in an increased rate of development of intestinal morphology or augmented populations of lymphocytes in the caecal tonsils.

Chapter 4

Administration of an In-Feed Probiotic To Alter the Caecal Microbiome

4.1 Introduction

Poultry probiotics are often derived from a limited selection of bacterial genera, including *Bifidobacterium* and *Lactobacillus* (Gadde *et al.*, 2017). These taxa are either microaerophilic or strict anaerobes with slow growth rates and a sensitivity to high temperatures. These characteristics present practical and logistical obstacles when considering mass production and delivery of vegetative cells to commercial poultry (Grant *et al.*, 2018). *Bacillus* are another commonly used genera which overcome these problems by producing spores resistant to high temperatures and oxygen (Grant *et al.*, 2018). Additionally, these spores can survive the acid conditions of the stomach and germinate in the small intestine of chickens (Cartman *et al.*, 2008). There are many examples in the literature of studies investigating the beneficial impact of *Bacillus* in-feed probiotics on disease resistance, immune development and performance parameters (Gadde *et al.*, 2017; Grant *et al.*, 2018). However, less is known about the mechanism of action of probiotics. One proposed mechanism is that beneficial microbes are able, in some way, to modulate or stabilise the composition of the intestinal microbiome and so exert their positive effect. Despite this proposed mechanism less work has been conducted to observe the effect of probiotic supplementation on the composition of the intestinal microbiome.

Strains of *Bacillus amyloliquefaciens* (BA) are less commonly used as commercial

probiotics than other similar species like *B. licheniformis* or *B. subtilis*. Many studies discussing the effects of administering BA to poultry do not define the origin of the strain used, perhaps with the exception of one study where the strain was isolated from the environment (Geeraerts *et al.*, 2015). Instead, authors focus on the *in vitro* properties of the species. Strains of BA have been shown to produce α-amylase and cellulase (Lee *et al.*, 2008; Gould *et al.*, 1975). It has been suggested that this production of enzymes could increase nutrient availability with dietary supplementation of α-amylase to broilers shown to increase nutrient digestibility (Gracias *et al.*, 2003). While the α-amylase used in that study was derived from a BA strain it was not produced by BA within the gut but produced exogenously and administered in the diet. Another potentially beneficial product of BA are bacteriocins with bactericidal effects against potential pathogens such as *Salmonella* Enteritidis and *Escherichia coli* (Lisboa *et al.*, 2006). In terms of modulation of the intestinal microbiome, some studies have observed the effect of BA supplementation on the caecal microbiome although these observations are often secondary to performance parameters and nutrient digestibility. The majority of these studies have relied on culture to enumerate bacterial populations in the caecum. Two studies by the same authors have reported the same result, that oral supplementation with BA led to reduced caecal *E. coli* and increased *Lactobacillus* (Lei *et al.*, 2014, 2015). Whilst the benefits of reducing *E. coli* are well known the authors do not explain why an increase in caecal *Lactobacillus* should be considered a positive outcome, especially considering that 16S rRNA gene sequencing has revealed the relatively minor role that *Lactobacillus* plays in the caecal microbiome compared to other taxa. However, a similar observation of reduced *E. coli* and increased *Lactobacillus* in the ileum, where *Lactobacillus* is a cornerstone of the microbiome, has been observed with oral administration of *B. subtilis* (Wu *et al.*, 2011). Reductions in *E. coli* populations in the caecum have also been reported without an increase in *Lactobacillus* (Ahmed *et al.*, 2014) calling into question whether increases in caecal *Lactobacillus* could be responsible for the observed decreases in *E. coli*. Tsukahara *et al.* (2017), used qPCR to measure the abundance of specific taxa in the ilea and caeca of probiotic treated chickens that had also been challenged with *Eimeria*. The authors claim that BA administration reduced

Clostridium perfringens and *E. coli* colonisation and increased *Lactobacillus* abundance. However, *Lactobacillus* abundance was only higher in probiotic treated chicks at one of three time points and then only in the ileum. Equally, *E. coli* and *C. perfringens* abundance was lower in probiotic treated chicks at one of three time points which, in the case of *E. coli*, did not coincide with the increased population of *Lactobacillus*. 16S rRNA gene sequencing revealed that the relative abundance of some genera of Lachnospiraceae and Ruminococcaceae was increased in probiotic chicks compared to control chicks (Tsukahara *et al.*, 2017) although the study lacks a description of the statistical methodology used to determine differentially abundant taxa. No correlation between qPCR results for *E. coli* or *C. perfringens* and their relative abundance on 16S rRNA gene sequencing results was mentioned by the authors. One other study has observed the effect of BA supplementation on the caecal microbiome using 16S rRNA gene sequencing. The results of this study agree to a certain extent with those mentioned previously in that the authors noted an increased relative abundance of Ruminococcaceae (specifically *Faecalibacterium* and *Blautia*) and Rikenellaceae in probiotic treated chickens. However, there was no report of decreased *E. coli* abundance but rather probiotic treated chicks had significantly more *Enterobacteriaceae* than controls at one time point. While the authors claim that beta diversity was significantly affected by BA administration, no statistical tests are provided to substantiate the claim and examination of UPGMA trees and PCoA plots of weighted UniFrac distances reveals no convincing effect of treatment on beta diversity (Cao *et al.*, 2018).

While the aforementioned studies provide some evidence that BA could have a role in modulating the composition of the caecal and ileal microbiomes, the methodologies used and statistical interpretation of the data introduce elements of doubt regarding the accuracy of the claims made. Equally, it should be questioned whether a single probiotic taxon could exert enough influence to change the composition of such a complex ecosystem as the intestinal microbiome.

The aim of this study was to observe the effect on the caecal microbiota of in-feed administration of BA spores. Most of the studies considered in the introduction have not attempted to recover BA from the ileal or caecal content so this study also aimed to

establish the extent to which this taxon can colonise the chicken gastrointestinal tract.

4.2 Materials and Methods

The experiment was run in two trials (Trial One and Trial Two), each lasting for 14 days and conducted one week apart. Each trial included a treated group which received in-feed probiotic and a negative control group.

4.2.1 Animals and housing

52 (Trial One) and 51 (Trial Two) day-old chicks were purchased from a commercial hatchery (Annyalla Chicks, Wrexham). On arrival, five chicks were sampled and the remaining chicks divided into two groups. The control group, composed of 24 chicks in Trial One and 23 chicks in Trial Two, received a pelleted vegetable protein based starter diet supplemented with 750 FTU/kg of phytase (Axtra® PHY B, Danisco Animal Nutrition). The treated group, composed of 23 chicks in Trial One and 23 chicks in Trial Two, received the control diet further supplemented with 1200 U of xylanase, 150 U of β-glucanase (PD Axtra® XB 201 TPT, Danisco Animal Nutrition) and 1.5 x 10^8 CFU/kg of BA 15AP4, BS8 and 2084 (Enviva Pro 202 BA, Danisco Animal Nutrition). The supplements were supplied by DuPont Industrial Biosciences and the diet was formulated by the Special Diets Service, Essex. The nutritional composition of the base diet is provided in Appendix C. Control and treated groups were housed in separate rooms in brooder pens with a wood shaving substrate. Feed and water were provided *ad libitum* by a drinker and feeder present in each brooder. Feed was weighed every day and average feed consumption per chick calculated. Five chicks from each group were sampled at 3, 7 and 11 d.p.h with all remaining chicks sampled at 14 d.p.h. One chick from the treated group in Trial Two died unexpectedly during the experiment at 7 d.p.h but the cause of death was not determined.

4.2.2 Sample collection

Samples were taken at 0, 3, 7, 11 and 14 d.p.h. Birds were weighed subsequent to euthanasia by cervical dislocation. At 0 d.p.h, only the caecal content was sampled since results from Chapters 2 and 3 showed that there was little difference between the caeca and ilea at 0 d.p.h and the microbiome was largely homogenous between chicks. Otherwise, the same sampling protocol as described in Chapter 3.2.3 was used.

4.2.3 DNA extraction

DNA was extracted from caecal and ileal contents using the same protocol described in Chapter 3.2.4.

4.2.4 Illumina MiSeq sequencing

Extracted DNA from all caecal samples from 3, 7 and 11 d.p.h and five caecal samples from each group at 14 d.p.h were sent for Illumina MiSeq sequencing. Extracted DNA from ileal samples taken at 14 d.p.h and caecal samples taken at 0 d.p.h in Trials One and Two were pooled for sequencing. Rather than pooling gross samples as in Chapter 2, extracted DNA was pooled as follows. The concentration of DNA in each constituent sample was measured using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies). The DNA concentration was used to calculate the volume required for pooling such that the same amount of extracted DNA was added from each constituent sample. The amount of DNA for pooling was based off the constituent sample with the lowest DNA concentration and therefore varied between groups. 800ng of DNA was pooled from each 0 d.p.h caecal sample from Trial One giving a final DNA concentration of 40ng/µl. 2,000ng of DNA was pooled from each 0 d.p.h caecal sample from Trial Two giving a final DNA concentration of 100ng/µl. 4,000ng of DNA was pooled from each ileal sample taken at 14 d.p.h giving a final DNA concentration of 200ng/µl. Extracted DNA was sent for paired-end sequencing of the 16S rRNA gene at the Centre for Genomic Research (University of Liverpool) as described in Chapter 2.2.4.

4.2.5 Amplicon sequence variant identification and taxonomy assignment

QIIME2 version 2019.1.0 was used for analysis of the Illumina data (Bolyen *et al.*, 2019) and conducted as described in Chapter 2.2.5

4.2.6 Data analysis

Diversity analyses, production of taxa plots and identification of differentially abundant taxa was conducted as described in Chapter 2.2.6 with alpha and beta diversity analyses performed at a sampling depth of 6,000.

4.2.7 Statistics

For statistical analysis, pooled 0 d.p.h caecal samples and 14 d.p.h ileal samples were removed. Samples were grouped according to age, treatment group and trial creating 16 different groups for comparison: samples from Trial One (T1) from control (C) and treated (T) chicks at 3, 7, 11 and 14 d.p.h (T1_C03, n=5; T1_T03, n=5; T1_C07, n=5; T1_T07, n=5; T1_C11, n=5; T1_T11, n=5; T1_C14, n=5; T1_T14, n=5), samples from Trial Two (T2) from control (C) and treated (T) chicks at 3, 7, 11 and 14 d.p.h (T2_C03, n=5; T2_T03, n=5; T2_C07, n=5; T2_T07, n=5; T2_C11, n=5; T2_T11, n=5; T2_C14, n=5; T2_T14, n=5). Statistical analysis between groups for alpha diversity, beta diversity and Gneiss analysis was conducted as described in Chapter 2.2.6.

4.2.8 Quantitative PCR

Quantitative PCR was conducted on ileal samples from 3, 7, 11 and 14 d.p.h using the same protocol described in Chapter 3.2.11. Primers used are displayed in Table 4.1.

4.2.9 Culture of BA from pelleted feed

1g of control and treatment diet were homogenised in 10ml phosphate buffered saline using a Stomacher® 80 paddle blender (Seward, Worthing, UK). 1ml of homogenised diet mixture was added to 4ml of buffered peptone water (pH 7.2, LabM) and incubated in an

Target Taxa	Primers	Amplicon Size (b.p.)	Reference
Domain *Bacteria* (targets V4 region)	F: TGCCAGCMGCCGCGGTAA R: GGACTACHVGGGTWTCTAAT	254	(Caporaso *et al.*, 2012)
Clostridium	F: TGCCAGCMGCCGCGGTAA R: GGACTACHVGGGTWTCTAAT	131	(Amit-Romach *et al.*, 2004)
Enterobacteriaceae	F: GTGCCAGCMGCCGCGGTAA R: GCCTCAAGGGCACAACCTCCAAG	429	(Smith *et al.*, 2014*b*)
Candidatus *Arthromitus*	F: GATGCGTAGGCGGTTGAGTA R: GGGTTTCTAATCCTGTTTGCTCC	90	This study
Lactobacillus	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	341	(Walter *et al.*, 2001) (Heilig *et al.*, 2002)
Enterococcus	F: CCCTTATTGTTAGTTGCCATCATT R: ACTCGTTGTACTTCCCATTGT	144	(Rinttila *et al.*, 2004)

Table 4.1: Primer pairs used for quantitative PCR in ileal samples

an anaerobic incubater (Don Whitley Scientific, Bingley, UK) at 37°C for 12 hours. 5µl of the incubated mixture was plated onto brain heart infusion (BHI) agar (Sigma-Aldrich Company Ltd, Gillingham, UK) and incubated under the same anaerobic conditions for 24 hours. After 24 hours, single colonies were subcultured onto BHI agar plates for a further 24 hours to obtain pure cultures. DNA was extracted from cultured bacteria using a boil preparation. Briefly, cultured bacteria were suspended in 500µl of nuclease free water (Thermofisher Scientific, Warrington, UK). The bacterial suspension was heated to 95°C for 15 minutes then allowed to cool to room temperature. Once cool, the suspension was centrifuged at 10,000 rpm for 5 mins. The supernatant was removed without disturbing the solid pellet and used as template DNA in a PCR to detect BA as described below.

4.2.10 PCR to detect BA DNA

Extracted DNA from three ileal and three caecal samples from treated chicks at each time point over both trials and one ileal and one caecal sample from a control chick from each time point over both trials were tested for the presence of BA using PCR. The PCR mixture was composed of 5µl of 5x FIREPol Master Mix Ready to Load (Solis BioDyne, Estonia), 1µl of each primer, 17µl of purified water and 1µl of DNA template. A primer pair specific for BA (F: GGCGGGAGGAAGTTAGGTGTA, R: CAGCATACCGGCAAAGCA) was used (primer sequences supplied by the manufacturer of Enviva Pro 202 BA). DNA

extracted from cultured BA was used as a positive control. Thermal cycling consisted of an initial cycle of 95°C for 5 min, 30 cycles of 95°C for 30s, 60°C for 45s and 72°C for 60s followed by a final cycle of 72°C for 5 mins. The presence of PCR products was confirmed by electrophoresis using a 1.0% agarose gel containing ethidium bromide.

4.3 Results

4.3.1 Body weight and feed consumption

The average body weight of day old chicks was 44g with no significant differences between Trials One and Two. Body weight increased with age with chicks attaining an average body weight of 546g by 14 d.p.h (Figure 4.1a). There were no significant differences in body weight between treatments or trials in chicks of the same age.

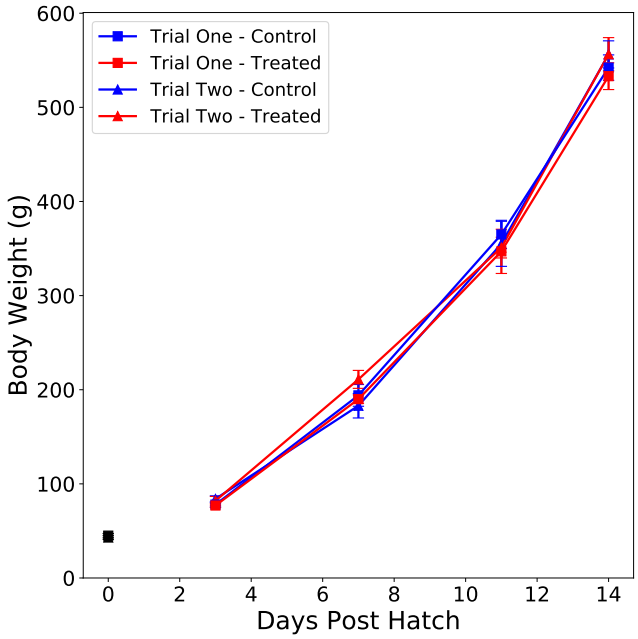
Feed consumption increased with age. The feed consumption of control chicks increased linearly, however, the feed consumption of treated chicks was more variable. In both trials, the average feed consumption of treated chicks increased at 9 d.p.h although the increase in feed consumption cannot be described as significant compared to controls due to small sample size (Figure 4.1b).

4.3.2 Sequencing effort

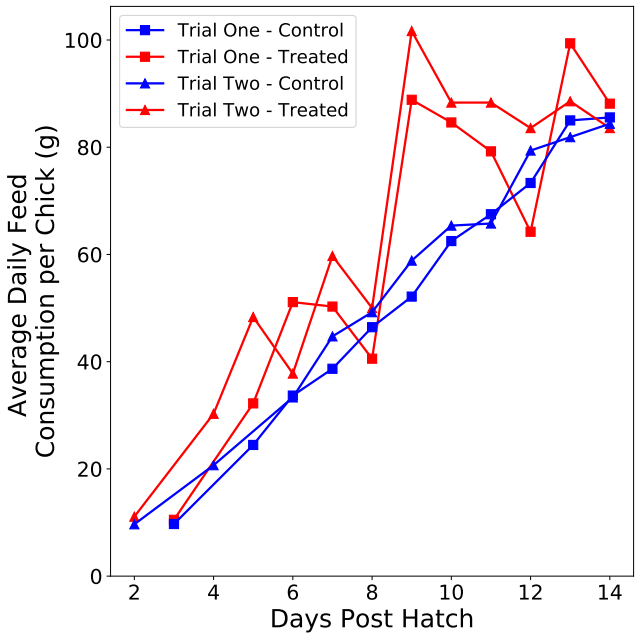
A total of 9,843,160 reads were obtained from 96 experimental samples submitted for sequencing. After filtering, merging of paired reads and chimera removal, a total of 7,524,113 reads remained (76% of the original total) giving a mean of 78,376 reads per sample. The median number of reads per sample was 74,725.

4.3.3 Alpha diversity

In Trial One, the pooled sample from 0 d.p.h had a low alpha diversity (Figure 4.2) when measured by both FPD and SD indices, although statistical comparisons between Trials and with later time points cannot be made due to the small sample size. In Trial Two, the pooled sample from 0 d.p.h had a high FPD index although the SD index continued to



(a) Average chick body weight at sampling in Trials One and Two



(b) Average daily feed consumption per chick in Trials One and Two

Figure 4.1: Average chick body weights and feed consumption

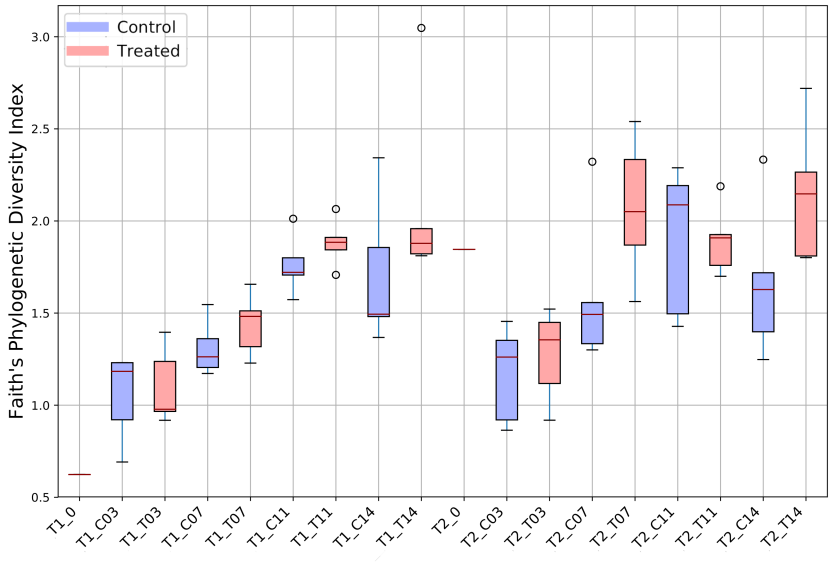
Error bars indicate the standard deviation of body weight

be low suggesting that although different taxa were detected in this sample there was low evenness. There were no significant differences in alpha diversity between samples taken at 3 d.p.h between trials or treatment groups. There was a significant increase in FPD index between 3 and 7 d.p.h in both treatment groups across both trials ($H = 13.9$, $p < 0.001$). However, there was no significant increase in SD between 3 and 7 d.p.h ($H = 0.80$, $p = 0.41$) suggesting that the increase in phylogenetic diversity was not accompanied by an increased evenness. At 7 d.p.h there was no effect of treatment or trial on FPD or SD. There was a significant increase in both FPD and SD between 7 and 11 d.p.h ($H = 7.61$, $p = 0.01$ and $H = 11.61$, $p = 0.002$ respectively). At 11 d.p.h, there were no significant differences between treatment groups in either FPD or SD indices. Between 11 and 14 d.p.h, there were no increases in FPD or SD alpha diversity nor were there any differences in these two indices between treatment groups at 14 d.p.h.

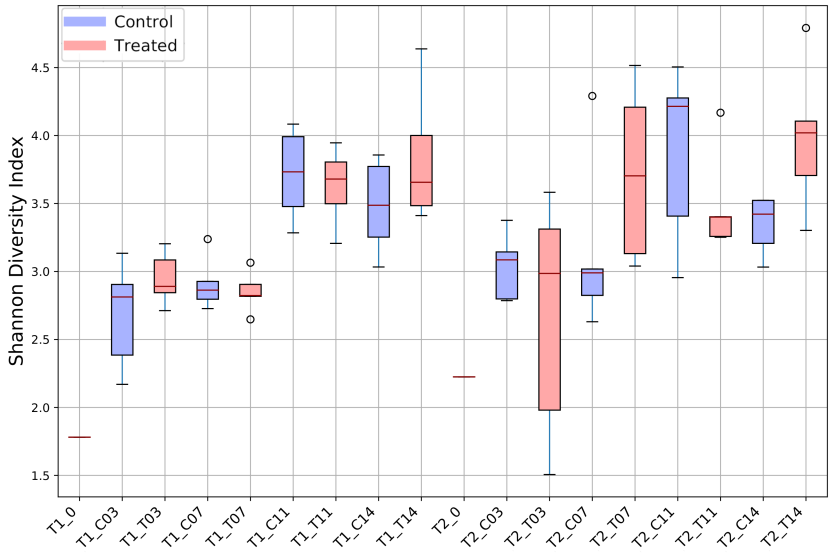
4.3.4 Beta diversity

Beta diversity was examined using both weighted and unweighted UniFrac metrics. Using an unweighted UniFrac metric, age had the largest effect on beta diversity (ANOSIM test statistic = 0.28, $p = 0.001$) followed by treatment group (ANOSIM test statistic = 0.20, $p = 0.001$) and trial (ANOSIM test statistic = 0.11, $p = 0.001$). In PCoA plots, some clustering by treatment group was evident from 7 d.p.h (Figure 4.3a). At 3 d.p.h, T1_C03 and T1_T03 samples clustered together along with most samples from T2_C03. However, samples from T2_T03 clustered separately from other samples taken at the same time point with two samples from this group showing some similarity to samples from later time points. At 7 d.p.h, there was more obvious clustering of samples by treatment group with most control samples from both trials clustering together while treated samples from both trials formed another cluster. This pattern was present, but less well defined, at 11 and 14 d.p.h as some control samples joined the same cluster as treated samples.

Using a weighted UniFrac metric, age had the largest effect on beta diversity (ANOSIM test statistic = 0.29, $p = 0.001$) followed by trial (ANOSIM test statistic = 0.05, $p = 0.009$). Treatment did not have a significant effect on beta diversity (ANOSIM test statistic = 0.01,



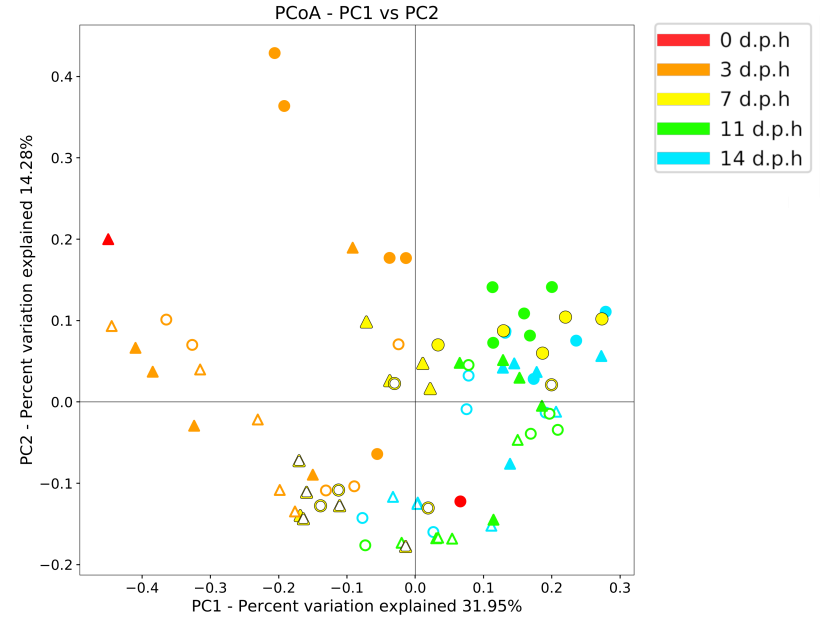
(a) FPD Index



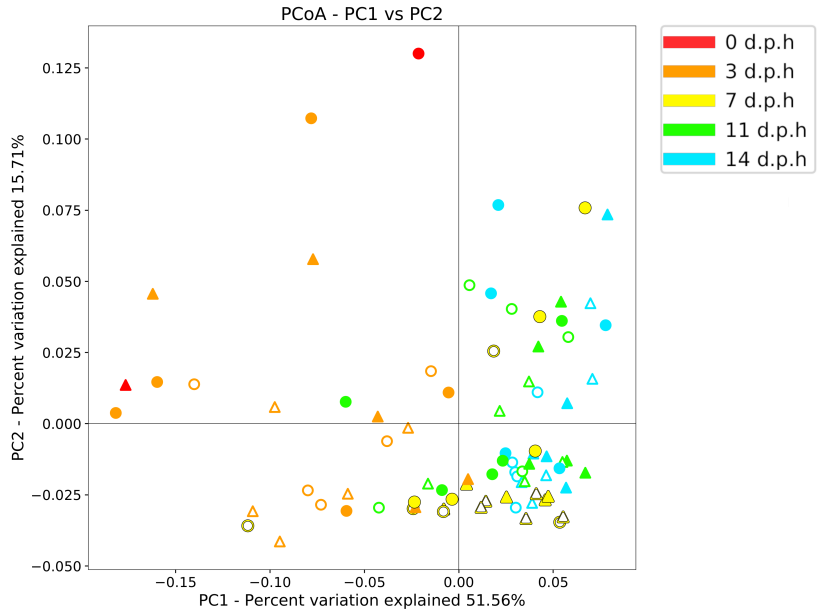
(b) SD Index

Figure 4.2: Alpha diversity of caecal samples in Trials One and Two

p = 0.11). When samples were viewed by treatment group in PCoA plots there was no pattern of clustering between treated and control samples at any time point (Figure 4.3b).



(a) Unweighted UniFrac



(b) Weighted UniFrac

Figure 4.3: Beta diversity between caecal samples in Trials One and Two

Sample metadata is identified by age (color), treatment (Control: No fill; Treated: Filled) and experiment (Trial One: ▲ ; Trial Two: ●). Each point represents a sample with distance between points representative of differences in microbiome composition.

4.3.5 Taxonomic composition

At 0 d.p.h, the caeca had a limited diversity as described in previous chapters. The pooled sample from Trial One was mainly composed of Enterobacteriaceae while that from Trial Two was composed of Clostridiaceae 1 (Figure 4.4). In samples from 3 d.p.h, Enterobacteriaceae was the most common taxon in the majority of samples. Lachnospiraceae was the second most abundant taxon and was the most abundant in samples where Enterobacteriaceae was not. Other taxa were also present, including Enterococcaceae, Lactobacillaceae, Ruminococcaceae and Clostridiaceae 1. A low abundance of Paenibacillaceae was noted in a few samples.

Samples from 7, 11 and 14 d.p.h had a similar taxonomic composition. The most abundant taxon in the majority of samples was Lachnospiraceae. Enterobacteriaceae was less abundant than at 3 d.p.h but continued as a major constituent of the caecal microbiome. The relative abundance of Ruminococcaceae increased compared to 3 d.p.h and formed part of the microbiome in all samples. The presence of other taxa was not consistent across all samples. Other notable constituents of the caecal microbiome were Enterococcaceae, Clostridiaceae 1, Clostridiales vadin BB60 group and Lactobacillaceae. A small abundance of Erysipelotrichaceae was present from 11 d.p.h in some caecal samples and Peptostreptococcaceae was present from 14 d.p.h.

Taxa plots of pooled ileal sample composition was used to select taxa for measurement of abundance using quantitative PCR. Enterococcaceae and Enterobacteriaceae were present in all pooled ileal samples. Clostridiaceae 1 were also present in all samples. Since both *Clostridium sensu stricto* 1 and *Candidatus* Arthromitus belong to this family, the data was examined at the genus level. All pooled ileal samples contained *Clostridium sensu stricto* 1 and *Candidatus* Arthromitus was only present in the pooled sample from control chicks in Trial Two. Given *Candidatus* Arthromitus' importance in the ileal microbiome, this taxon was selected for quantitative PCR. Equally Lactobacillaceae was only detected in the pooled sample from treated chicks in Trial Two but given this taxon's importance it was also selected for quantitative PCR. Peptostreptococcaceae had a high relative abundance in

the pooled sample from control chicks in Trial One. At the genus level these were further identified as *Romboutsia*, however, due to the lack of published specific primers for this genus it was not selected for quantitative PCR.

4.3.6 Differentially abundant taxa between treated and control chicks

Gneiss analysis revealed differential ASV abundance between caecal samples from treated and control chicks between 3 and 14 d.p.h. The ASV table was filtered to exclude ASVs with a frequency of less than 924 reducing the number of ASVs in the analysis from 212 to 107. The overall linear regression model fit was R2 = 0.234 with covariate 'Treatment' accounting for 5.95% of variance. Other variants included in the regression model were 'Trial' (accounting for 7.2% of variance) and 'Age' (account for 10% of variance). Balances y0 (β= -5.27, p <0.001), y6 (β= 4.15, p <0.001), y7 (β= -2.23, p <0.001), y12 (β= -2.51, p = 0.002), y18 (β= -1.37, p = 0.03) and y23 (β= 2.39, p = 0.007) were significant predictors for the covariate 'Treatment'. On review of the dendrogram heatmap (Figure 4.5), balance y1 allowed greater resolution on ASVs which were differentially abundant between treatment groups despite not being a significant predictor for the covariate 'Treatment', (β= -1.47, p = 0.27). Individual log ratios by sample group for significant balances and balance taxonomy are available in Figure C.1 and Table C.1 respectively.

Briefly, the log ratio of balance y0 was lower in treated samples. The dendrogram heatmap showed that a subdivision of ASVs in $y0_{denominator}$ were equally abundant between sample groups while others had a higher log abundance in some samples from treated chicks in Trial Two at 7, 11 and 14 d.p.h. Those with a higher log abundance were defined by balance $y1_{denominator}$. Balance y6 is a subdivision of balance $y0_{numerator}$ and was lower in control samples with the dendrogram heatmap showing a higher log abundance of $y6_{denominator}$ in control samples. Balance y12 was lower in treated samples from Trial One at 11 and 14 d.p.h. The dendrogram heatmap shows a higher relative abundance of $y12_{denominator}$ ASVs in these samples. Other balances that were significantly different between treatment groups were subdivisions of balances already identified as describing differentially abundant ASVs.

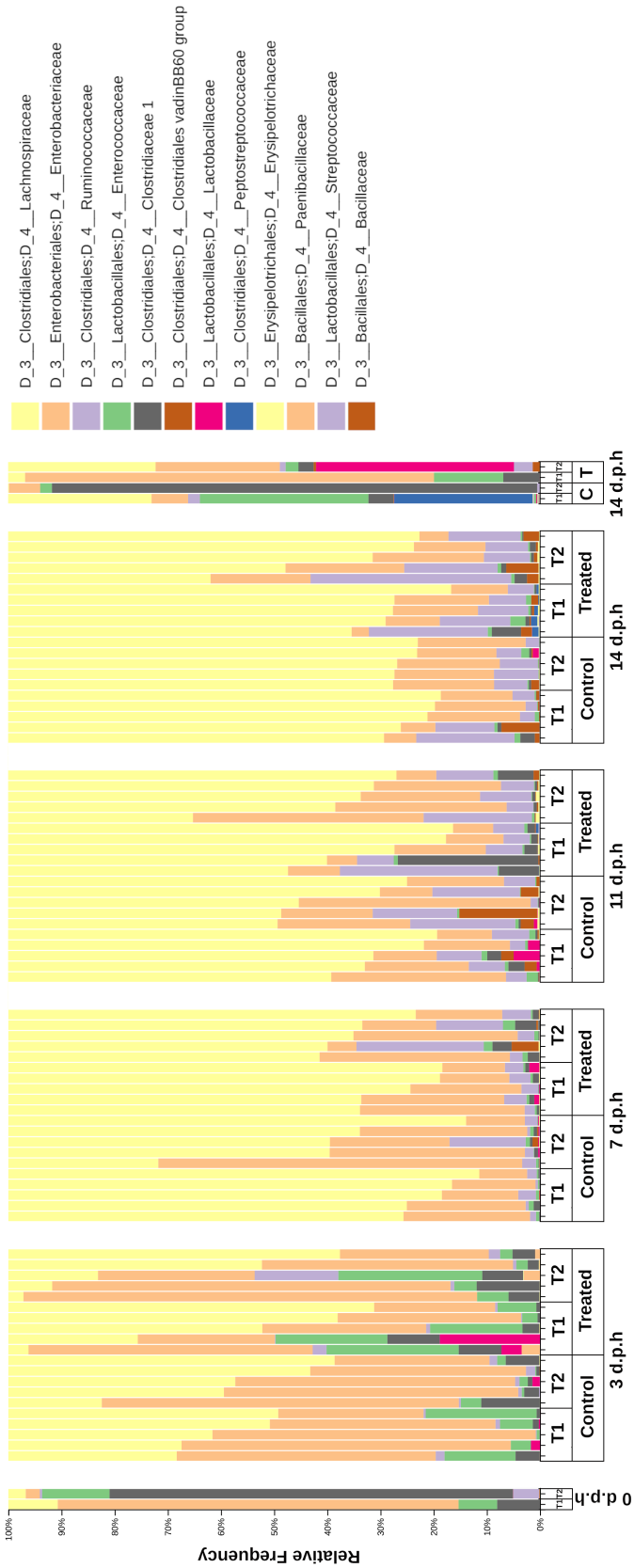


Figure 4.4: Relative abundance of bacterial families in the caeca and ilea of treated and control chicks between 0 and 14 d.p.h in Trials and Two

Trial One (T1); Trial Two (T2)

		Number of ASVs		
Taxonomy	**Total**	**Treated**	**Control**	**NDA** [a]
Lachnospiraceae	15	2	7	6
Clostridium sensu stricto 1	9	0	0	9
[Ruminococcus] torques group	7	2	2	3
Ruminococcaceae UCG-014	6	3	0	3
Ruminococcaceae	5	1	3	1
Escherichia-Shigella	5	0	0	5
Enterobacteriaceae	4	0	0	4
Ruminiclostridium 5	4	0	2	2
uncultured	4	2	2	0
Sellimonas	3	2	0	1
Flavonifractor	3	1	1	1
Enterococcus	3	0	0	3
[Eubacterium] coprostanoligenes group	2	1	1	0
Paenibacillus	2	0	0	2
Eisenbergiella	2	0	1	1
Lachnoclostridium	2	0	0	2
Pediococcus	2	0	0	2
uncultured bacterium	2	0	0	2
Butyricicoccus	2	0	2	0
Erysipelatoclostridium	2	0	2	0
Subdoligranulum	2	0	2	0
Anaerostipes	1	0	0	1
Proteus	1	1	0	0
Blautia	1	0	1	0
Lactobacillus	1	1	0	0
Epulopiscium	1	0	0	1
Hydrogenoanaerobacterium	1	1	0	0
CHKCI001	1	1	0	0
Romboutsia	1	0	1	0
ASF356	1	0	1	0
Oscillibacter	1	0	0	1
Ruminiclostridium 9	1	0	1	0
Anaerotruncus	1	0	1	0
Lachnospiraceae NK4A136 group	1	1	0	0
Shuttleworthia	1	0	0	1
Tyzzerella 3	1	1	0	0
Ruminococcaceae UCG-013	1	1	0	0
Caproiciproducens	1	0	0	1
DTU089	1	0	0	1
Streptococcus	1	1	0	0
GCA-900066225	1	0	0	1
CAG-56	1	1	0	0

[a] ASVs defined as NDA were not differentially abundant between caecal and ileal samples. Individual taxonomies of significant Gneiss balances are provided in Table C.1.

Table 4.2: Taxonomy of differentially abundant ASVs between treated and control samples

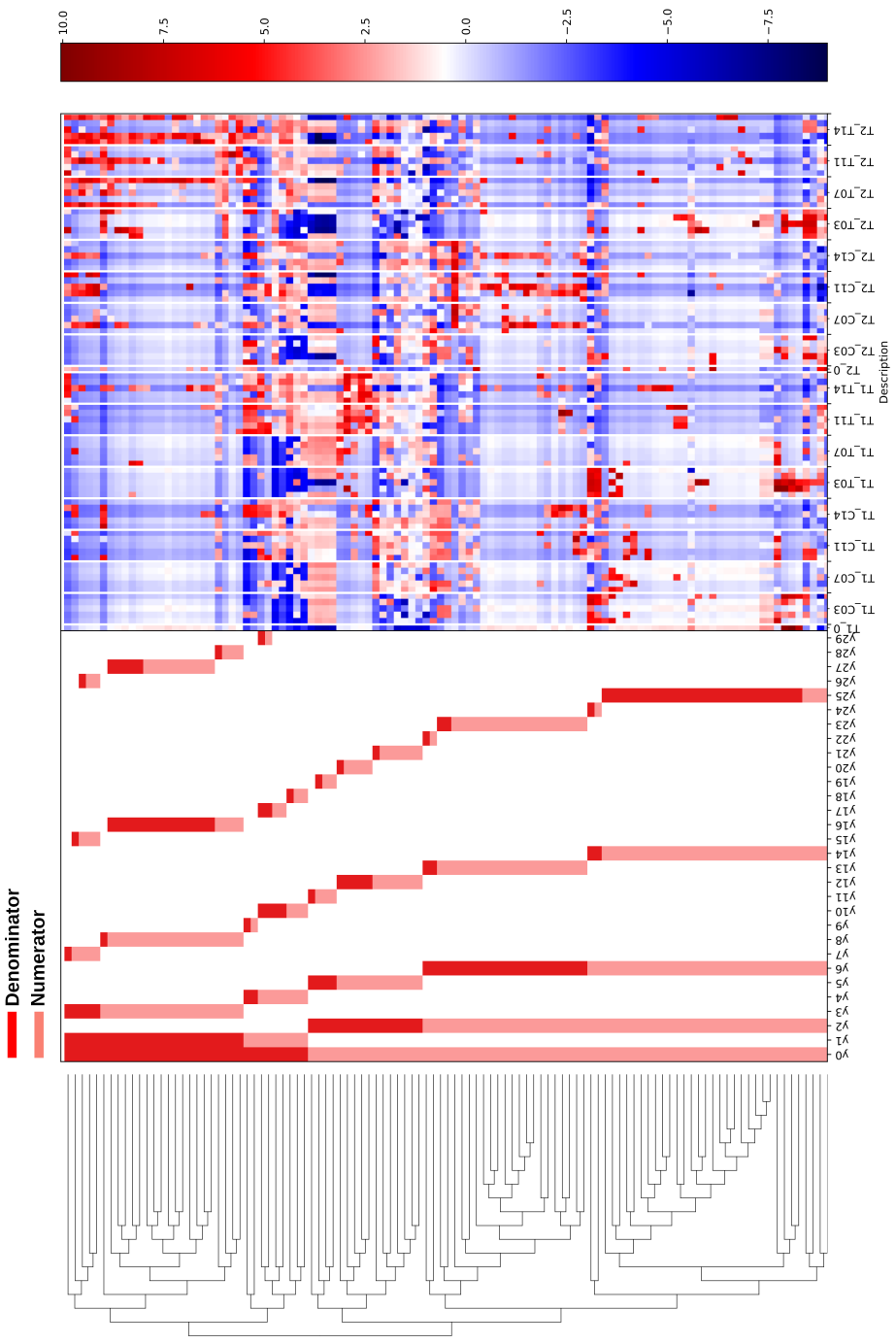


Figure 4.5: Dendrogram heatmap of ASV abundance showing differences in caecal microbiome composition between treated and control chicks

A dendrogram heatmap showing the log abundance of ASVs in caecal samples grouped by trial, treatment and age. Differences in relative abundance between treatments are visible in balances y1, y6 and y12 identified by Gneiss analysis as containing differentially abundant ASVs.

However, as previously noted, the dendrogram heatmap shows that balances which were significantly different between treatment groups had higher log ratios in treated samples from either Trial One or Two, with no ASVs consistently differentially abundant in treated samples between Trials. This is confirmed by the regression analysis where balances y0 (β= -6.15, p <0.001) and y12 (β= 2.26, p = 0.006) were also significantly affected by 'Trial'. Overall, since 'Trial' accounted for 7.2% of total variance compared to 'Treatment' which account for 5.95%, it can be concluded that treatment did not have a greater effect on caecal microbiome composition than trial.

4.3.7 Detecting the presence of BA

Identifying sequences assigned to *Bacillus*

Four ASVs were assigned to the genus *Bacillus*. These sequences were detected in five samples. The frequency of each sequence in these samples is shown in Table 4.3. Three *Bacillus* ASVs were found in pooled samples from control ileum at 14 d.p.h and caecum at 0 d.p.h suggesting that these were not derived from in-feed probiotics. One *Bacillus* ASV, 08816152c731dc4bb29fcd5e072fe7dc (ASV-0881), had a low frequency in the pooled ileal sample from treated chicks in Trial Two. This ASV may represent DNA recovered from vegetative cells of the in-feed probiotic.

Sequences of *Bacillus* ASVs were searched against known BA sequences available in the NCBI 16S rRNA gene database using BLAST. Two ASVs, ASV-0881 and 5cd16041435cc76f56d83f-ba8857af6c (ASV-5cd1), had a 100% identity match to sequences from BA. ASV-5cd1 was found in ileal samples from control chicks in Trial One and can therefore be classified as an environmental bacteria rather than derived from the in-feed probiotic. Since BA sequences were recovered from both control and treated chicks, there is no guarantee that ASV-0881 was not derived from the environment as ASV-5cd1 was.

Sample Metadata	**Location**	Ileum	Ileum	Caecum	Caecum	Caecum
	Trial	One	Two	Two	Two	Two
	Treatment Group	Control	Treated	N/A	Control	Control
	Age (d.p.h)	14	14	0	14	14
ASV ID	08816152c731dc4bb29fcd5e072fe7dc	0	6	0	0	0
	fb134d9cec9f0eb150bb1ca1668c7a41	36	0	77	4	5
	5cd16041435cc76f56d83fba8857af6c	21	0	0	0	0
	1bd091ada6dfd4dfcb31070118180938	0	0	3	0	0

Table 4.3: ASVs assigned to *Bacillus* and their frequency counts

Culture of BA from the treatment diet

BA was successfully cultured from the treatment diet with significant growth observed on plates within 12 hours. Colonies were confirmed as the correct strain of BA by a positive PCR. Growth of bacterial colonies was noted on agar plates inoculated with control feed, however, none of these colonies was identified as BA using PCR. This suggests that the diet was successfully produced with viable BA spores present which could germinate under the correct stimulus to form vegetative cells.

Detection of BA DNA in caecal and ileal samples using PCR

All caecal and ileal samples selected for screening of BA from control and treated chicks in Trials One and Two were negative for the presence of BA using PCR. DNA from BA cultured from the diet was used as a positive control and returned a positive result suggesting that the negative results obtained from field samples were not due to failure of amplification.

4.3.8 Quantitative PCR from ileal samples

The abundance of Enterobacteriaceae, Lactobacillaceae, *Clostridium*, *Enterococcus* and *Candidatus* Arthromitus was measured in individual ileal samples taken between 3, 7, 11 and 14 d.p.h. While significant differences in the abundance of these taxa were found between treated and control chicks, these differences were not consistent across all time points nor between trials (Figure 4.6).

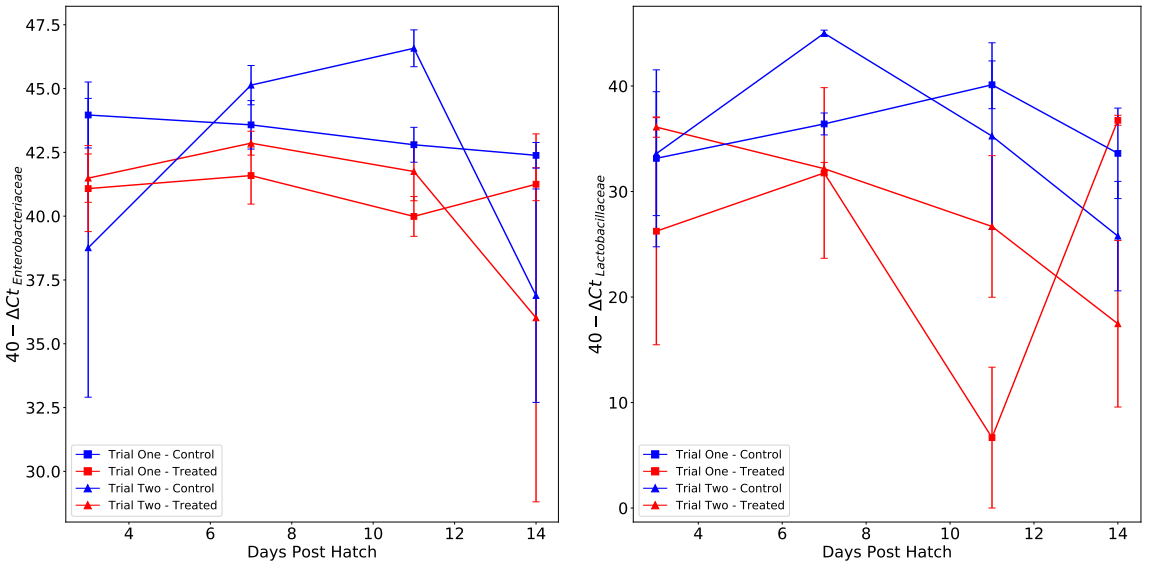
The abundance of Enterobacteriaceae had a tendency to decrease over time, although there was an increase in Enterobacteriaceae abundance in control chicks from Trial Two between 3 and 11 d.p.h. There were no significant differences in Enterobacteriaceae abundance between treated and control chicks except control chicks at 11 d.p.h in Trial Two which had a significantly higher abundance of Enterobacteriaceae than treated chicks (test statistic = 3.56, p = 0.05).

The abundance of Lactobacillaceae was more variable than that of Enterobacteriaceae with unpredictable increases and decreases over time. There was a significantly higher abundance of Lactobacillaceae in control samples at 11 d.p.h in Trial One (test statistic = 4.75, p = 0.01) and 7 d.p.h in Trial Two (test statistic = 19.4, p <0.001).

The abundance of *Clostridium* was similarly variable but no significant differences between treated and control chicks were found.

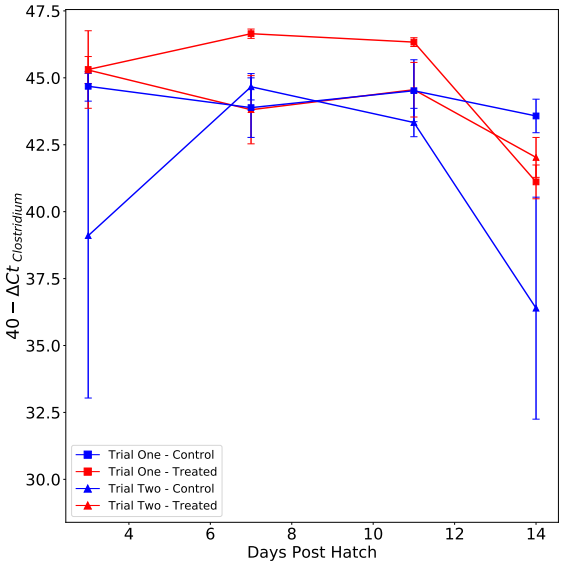
The abundance of *Enterococcus* was similar across all time points although low values were obtained for control chicks from Trial Two at 3 and 14 d.p.h. The abundance of *Enterococcus* in control chicks from Trial Two at 14 d.p.h was significantly lower than that of treated chicks (test statistic = 7.1, p <0.001).

Candidatus Arthromitus was not present in either treated or control samples from Trial One. In Trial Two, treated chicks exhibited strong colonisation from 7 d.p.h which was sustained over 11 and 14 d.p.h. In contrast, control chicks were colonised from 11 d.p.h with weaker colonisation. Consequently, treated chicks had a significantly higher abundance of *Candidatus* Arthromitus at 7 and 14 d.p.h in Trial Two (test statistic = -76.5, p <0.001 and test statistic = -3.5, p = 0.04 respectively).

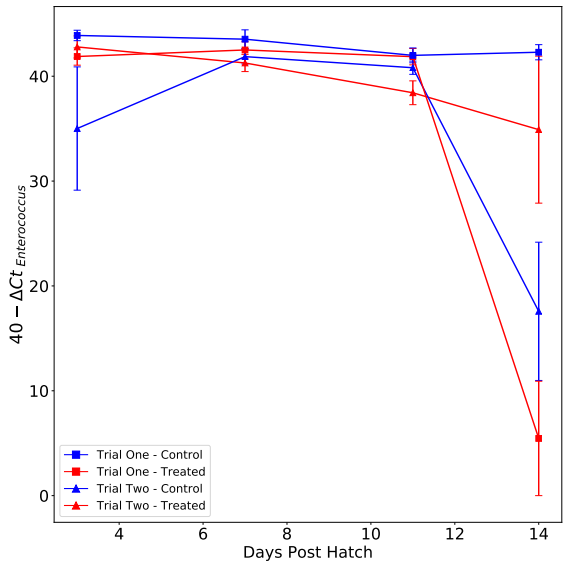


(a) Enterobacteriaceae

(b) Lactobacillaceae

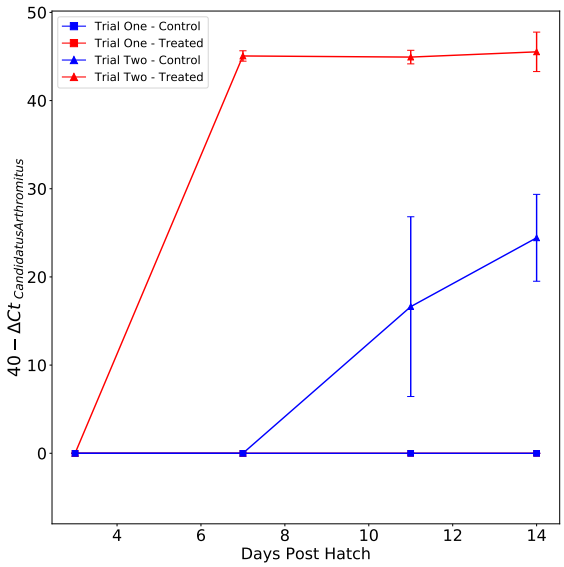


(c) *Clostridium*



(d) *Enterococcus*

Figure 4.6: Relative abundance of bacterial taxa in the ileum determined by quantitative PCR



(e) *Candidatus* Arthromitus

Figure 4.6: Relative abundance of bacterial taxa in the ileum determined by quantitative PCR

4.4 Discussion

The results of this study showed no effect of BA administration on the caecal or ileal microbiomes. Furthermore, analysis of 16S rRNA gene sequencing and PCR showed no appreciable colonisation by BA of either the caecum or ileum in treated chicks. This was despite successful bacterial culture of BA from the treatment diet demonstrating the viability of spores. However, it's not possible to conclude that BA was unable to colonise the gastrointestinal tract of the chickens entirely. This reluctance is driven by several factors. Firstly, sequences assigned to *Bacillus* were detected in treated samples which may represent sequences from BA. While 16S rRNA gene sequencing offers a comprehensive overview of the microbiota composition the technique is still limited by the number of sequence reads. Consequently, results should not be interpreted as a complete index of bacterial diversity and can only be used to confirm presence but not prove the absence of taxa. Secondly, the sensitivity of the PCR test used was not quantified. Although a positive result was achieved using DNA extracted from pure cultures of BA, it is possible that the PCR technique

coupled with visualisation of bands on agarose gel was insufficiently sensitive to detect small numbers of BA. Lastly, the biological behaviour of BA within the chicken gut has not been determined. It remains possible that this species successfully colonises more proximal areas of the gastrointestinal tract such as the duodenum or jejunum. Previous work in a mouse model has shown that *Bacillus subtilis* spores germinate and subsequently resporulate in the jejunum (Tam *et al.*, 2006; Casula and Cutting, 2002). However one other study has reported that no germination occurs in the mouse gastrointestinal tract (Spinosa *et al.*, 2000). Although Cartman *et al.* (2008) reported that vegetative cells of *B. subtilis* were detected in all parts of the gastrointestinal tract it should be taken into account that this observation was made after administration of spores to day-old chicks whose immature guts may not provide the correct environment to trigger resporulation. If BA behaves in the more mature chicken gut as *B. subtilis* does in the murine gut, there may have been minimal numbers of vegetative cells in the ileum and caecum. However, the possibility that the probiotic was unable to germinate and colonise the intestinal tract should not be excluded. Of the strains of BA used in the experiment, two were cultured from turkey litter and the provenance of one is not known (EFSA FEEDAP Panel, 2016). To date, the author is not aware of any publically available literature showing successful colonisation of the chicken gastrointestinal tract by these strains of BA.

There were no consistent differences in microbiome composition between treated and control chicks which could be attributed to probiotic administration. While differences between treated and control chicks were identified in the caecum by 16S rRNA gene sequencing these differences were not consistent between trials. Generally, ASVs that were identified as differentially abundant between treated and control chicks belonged to Lachnospiraceae and Ruminococcaceae while other taxa such as *Escherichia-Shigella* and *Clostridium sensu stricto* 1 were not differentially abundant between treatment groups. However, whether these differences were of functional significance is more difficult to determine. Similarly, there were significant differences in Lactobacillaceae and Enterococcaceae abundance in the ileum between treated and control chicks and one time point at which the difference in Enterobacteriaceae abundance between treated and control chicks was significant. *Candidatus*

Arthromitus abundance was significantly higher in treated chicks but only in Trial Two. However there was no significant difference in *Candidatus* Arthromitus abundance between treatment groups in Trial One since no *Candidatus* Arthromitus was detected in either group. In the context of the experimental setup these results are difficult to ascribe to the effect of the probiotic since there was no consistency in differences observed between treated and control chicks between trials. This difficulty in unravelling probiotic and environmental effects highlights a shortcoming in the approach to determining the effect of interventions on the intestinal microbiome. Although researchers go to great lengths to include an adequate number of replicates in their experiments it is not always clear to what degree replicates are spatially separated. This is of great importance when interpreting microbiome data due to the large impact that environmental factors have on microbiome composition. If replicates are inadequately separated spatially then the environment could account for any similarities between replicates or differences between treatment groups. The results derived from *Candidatus* Arthromitus provide a particularly good example. Even if a higher abundance of *Candidatus* Arthromitus were found in treated chicks in Trial One, since *Candidatus* Arthromitus survives as environmental spores it could be argued that the room in which treated chicks were housed had a higher initial abundance of spores than that of the control chicks. Ideally, quantitative PCR for bacterial genera such as *Turicibacter* and *Romboutsia* would have been conducted as these genera were identified in Chapter 2 as markers of a mature ileal microbiome. However, specific primers for these genera are not currently published in the literature.

Supplementation with BA had no effect on body weight although the experimental design used was not optimised to measure performance parameters. Additionally, this experiment only measured body weight until 14 d.p.h whereas other experiments reporting a positive effect of BA on performance parameters have encompassed the whole of the production period (Lei *et al.*, 2014; Tsukahara *et al.*, 2017). There were no significant differences in average daily feed consumption between treated and control chicks, however, while feed consumption increased linearly for control chicks, in both treated groups feed consumption increased and decreased sporadically. Other studies using the same BA

probiotic have noted a reduced feed consumption in treated groups (Grant *et al.*, 2018; Amerah *et al.*, 2013). No studies have examined the effect that probiotic inclusion has on feed palatability, but this may be a factor which influences the pattern of feed intake. While different patterns of feed intake didn't appear to have an effect on performance parameters, it would be worth questioning whether sporadic or reduced feed intake could have other effects on poultry health.

Although no effect of the probiotic was observed in this experiment it's possible that the effect of direct fed microbials is only evident during physiological stress or immunological challenge. One proposed mechanism by which probiotics function is providing a stabilising effect on the microbiome. Therefore, their effects would not be evident during a study of normal development but their presence may help to reduce the impact of insults such as colonisation by pathogens. Previous experiments reporting the positive effect of BA have used infection models to challenge chickens and observe whether BA reduces production loss or pathology (Tsukahara *et al.*, 2017; Lei *et al.*, 2015), although there are also reports in the literature of a BA probiotic failing to reduce the effect of necrotic enteritis on production (Jerzsele *et al.*, 2012). It's worth considering that modulation of the microbiome is only one proposed mechanism by which probiotics such as BA function. The lack of effect on the microbiome observed in this experiment does not negate previous research showing either a positive effect on intestinal morphology, performance parameters or disease resilience.

In summary, no effect on the development of the ileal or caecal microbiome was observed during administration of an in-feed BA probiotic between 0 and 14 d.p.h. Additionally, there was no significant effect of treatment on body weight of chickens at sampling between treated and control groups. These results suggest that previously reported positive effects of BA administration are not due to modulation of the microbiome.

Chapter 5

Discussion and Summary

This thesis has described the results of three experiments. The first, detailed in Chapter 2, aimed to set a baseline for the development of the caecal and ileal microbiomes by observing the development of the microbiome from 0 to 42 d.p.h. Secondary objectives were included to inform the methodology of subsequent experiments. These objectives included observing differences in the mucus and lumen associated microbiomes in the ileum and caecum, comparing the composition of pooled samples to that of constituent samples and comparing the effect of genotype on development and composition of the caecal and ileal microbiomes. Based on the results of this first experiment, a second, described in Chapter 3 was designed to observe the effect of topical application of adult caecal content to eggs on caecal and ileal microbiome development. Using the pattern of intestinal microbiome succession described in Chapter 2 as a template, this topical treatment resulted in accelerated development of the caecal and ileal microbiomes compared to a negative control group. The final experiment, described in Chapter 4, aimed to observe the effect of an in-feed multistrain probiotic on the development of the caecal and ileal microbiomes. In contrast to the results described in Chapter 3, no significant effect of probiotic administration on microbiome development was found in either the caecum or the ileum. Together, these three experiments provide evidence for a standard pattern of bacterial succession in the caecum and ileum which can be used to inform the composition, timing and mode of delivery for future interventions in the chicken intestinal microbiome.

5.1 The Pattern of Bacterial Succession in the Caecum and Ileum was Similar Across All Three Experiments

All three experiments covered the time period from hatch to 14 d.p.h with only the first experiment examining the composition of the microbiome until 42 d.p.h. As such, observations can only be made regarding the time period covered by all experiments.

5.1.1 Succession in the caecum

The post-hatch microbiome was similar across all three experiments. Although the exact taxonomic composition varied the caecal microbiome at hatch was composed of mainly one or two bacterial families. There was an abundance of potential pathogens with ASVs assigned to one or more of *Clostridium*, *Clostridium sensu stricto* 1 and *Escherichia/Shigella* identified in all three experiments. The genus *Enterococcus* was also present in two experiments. Although one strain of *Enterococcus cecorum* has been associated with spondylitis, *Enterococcus* is considered to be a normal commensal of the chicken gastrointestinal tract (Jung *et al.*, 2018). In one experiment, Chapter 3, *Paenibacillus* was present at a high relative abundance. Unlike the other taxa described at 0 d.p.h, *Paenibacillus* is not associated with the chicken intestinal microbiome although its presence in the trachael microbiome has been correlated with decreased body weight (Johnson *et al.*, 2018). In all experiments there was a small relative abundance of Lachnospiraceae and Ruminoccocaceae. Generally, the relative abundance of Lachnospiraceae was less than 3% although a higher relative abundance of 15% and 9% were noted in samples which had received no intervention in Chapters 2 and 4 respectively. The presence of Ruminococcaceae was less common than that of Lachnospiraceae with a commensurately lower relative abundance of less than 0.5%, although a relative abundance of 2.6% was recorded in one sample from Chapter 2.

The taxonomic composition of the caecal microbiome changed between 0 and 3 d.p.h in all experiments. There tended to be an increase in the relative abundance of *Escherichia/Shigella*, *Clostridium* and *Enterococcus*. Of the taxa that are associated with a

mature caecal microbiome, the relative abundance of Lachnospiraceae increased the most although the magnitude of the increase varied between experiments. For example, the relative abundance of Lachnospiraceae in control samples from Chapter 3 was less than 5%, in samples from Chapter 2 it increased to between 8 and 14% and in both treated and control samples from from Chapter 4 showed more variance with a relative abundance between 3 and 69%. The relative abundance of Ruminococcaceae remained below 3% in most samples across the three experiments. Samples from Chapter 2 showed the greatest taxonomic diversity as they were colonised by ASVs assigned to Bifidobacteriaceae and Burkholderiaceae which would continue to form a part of the caecal microbiome until 42 d.p.h.

Further changes occurred in all experiments between 3 and 7 d.p.h, although there were some differences between experiments. The hallmark of microbiota succession in Chapter 2 at 7 d.p.h was an increase in the relative abundance of Bacteroidaceae and a decrease in Enterobacteriaceae. Bacteroidaceae was not present in samples from Chapters 3 or 4. Instead, the principal change at 7 d.p.h was an increase in the relative abundance of Ruminococcaceae and, to a lesser degree, Lachnospiraceae which was also accompanied by a decrease in Enterobacteriaceae. The compositional nature of the data makes it difficult to interpret whether an increase in the abundance of Ruminococcaceae and Lachnospiraceae occurred in Chapter 2 but was obscured by the large increase in Bacteroidaceae abundance.

In all experiments a similar pattern of change was noted between 7 and 14 d.p.h. This was mainly marked by a continued increase in the relative abundance of Ruminococcaceae which was most noticeable in Chapters 2 and 4. Additional taxa were present in the microbiome by 14 d.p.h, such as Christensenellaceae, Clostridiales vadin BB60 group and Mollicutes RF39 although their relative abundance remained small in comparison to Bacteroidaceae, Ruminococcaceae and Lachnospiraceae. Only Chapter 2 continued past 14 d.p.h, but demonstrated that taxa such as Peptococcaceae and *Faecalibacterium prausnitzii* continued to join the microbiome until 42 d.p.h. It is likely that had other experiments been continued to the same time point further succession and increased diversity would have been observed.

These results demonstrate a consistent pattern of succession whereby species of Lachnospiraceae colonised the caecum first followed by members of Ruminococcaceae and subsequently other taxa like Christensenellaceae, Clostridiales vadin BB60 and Mollicutes RF39. Taxa such as Bacteroidaceae and Bifidobacteriaceae were only present in samples from Chapter 2 and as such no comment can be made as to how these taxa fit into a pattern of succession. As discussed in Chapter 2, some studies have shown that species of *Bacteroides* are able to metabolise exopolysaccharides produced by *Bifidobacterium* strains (Rios-Covian *et al.*, 2013; Salazar *et al.*, 2008) introducing the possibility that prior colonisation by Bifidobacteriaceae facilitated subsequent colonisation by Bacteroidaceae. However, further experiments observing succession and co-occurrence of these taxa in the caecum are required.

Other studies have also observed a similar pattern of succession in the caecum. A recent experiment by Glendinning *et al.* (2019) observed the development of the chicken intestinal microbiome from 1 d.p.h to 5 weeks old. A very low relative abundance of Lachnospiraceae and Ruminococcaeae was present at 1 d.p.h (0.004% and 0.003% respectively). At 3 d.p.h, the relative abundance of Lachnospiraceae increased to 26.4% while that of Ruminococcaceae remained low at 5%. Subsequently, the relative abundance of Ruminococcaceae increased until 5 weeks post hatch when its relative abundance was higher than that of Lachnospiraceae (35.7% and 29.7% respectively). Christensenellaceae, Clostridiales vadin BB60 and Mollicutes RF39 were not present in the caecum until 14 d.p.h, attaining their highest relative abundance at 5 weeks post hatch. Additionally, *Faecalibacterium prausnitzii* was associated with the caecal microbiome at 5 weeks post hatch as was observed in Chapter 2. Two further recent studies have shown initial caecal colonisation by Lachnospiraceae at around 3 to 4 d.p.h followed by an increase in Ruminococcaceae between 7 and 14 d.p.h (Jurburg *et al.*, 2019; Videnska *et al.*, 2014).

It is therefore reasonable to theorise that initial colonisation by Lachnospiraceae may facilitate caecal colonisation by Ruminococcaceae in some way. This was highlighted in Chapter 3 where colonisation of treated chicks by Lachnospiraceae occurred first despite the assured presence of Ruminococcaceae spores in the transplant material. However,

colonisation by Lachnospiraceae is unlikely to be a prerequisite for successful establishment of Ruminococcaceae in the caecum. This has been demonstrated in a recent experiment observing the effect of oral administration of a caecal fermentation broth on the development of the caecal microbiome in chicks (Gong *et al.*, 2019). The taxonomic composition of the broth was different to the caecal content administered in Chapter 3 with a lower relative abundance of Ruminococcaceae and no mention of Lachnospiraceae. The relative abundance of Ruminococcaceae in treated chicks was subsquently higher than that of Lachnospiraceae at 1, 3 and 7 d.p.h (Gong *et al.*, 2019). This suggests that the caecal environment is conducive to colonisation by Ruminococcaceae at hatch. One possible explanation for this discrepancy is the life-stage of Ruminococcaceae administered. Gong *et al.* (2019) administered vegetative Ruminococcaceae cells whereas the majority of Ruminococcaceae present in the transplant material used in Chapter 3 would be present as spores. It is therefore possible that Lachnospiraceae creates a caecal environment which stimulates germination of Ruminococcaceae spores rather than enabling colonisation by vegetative cells.

5.1.2 Succession in the ileum

Similar to the caecum, some elements of succession were shared in the two experiments which used 16S rRNA gene sequencing to observe the ileal microbiome. However, it should be noted that the most abundant taxon differed between experiments with samples described in Chapter 2 mainly colonised by Lactobacillaceae whilst those in Chapter 3 were mainly colonised by Enterococcaceae. In Chapter 2, Enterococcaceae was a prominent feature in the microbiome but was quickly replaced by Lactobacillaceae by 14 d.p.h. A similar pattern was described by Glendinning *et al.* (2019) with the relative abundance of Lactobacillaceae increasing at 14 d.p.h at the expense of Enterococcaceae. Similarly, *Enterococcus* has been described as a core member of the ileal microbiome at 0 d.p.h giving way to *Lactobacillus* between 7 and 21 d.p.h (Johnson *et al.*, 2018). It's possible that the sustained, high relative abundance of Enterococcaceae in Chapter 3 was due to the absence of environmental Lactobacillaceae until between 7 and 14 d.p.h. The influence this would

have had on host metabolism or the overall development of the microbiome is not known. While both Enterococcaceae and Lactobacillaceae produce lactic acid via fermentation detailed comparisons of their metabolic functions are lacking in the literature.

Common features between experiments included an increase in the relative abundance of *Candidatus* Arthromitus between 7 and 14 d.p.h. An increase in *Candidatus* Arthromitus abundance at this time period is a widely documented feature of the development of the ileal microbiome (Glendinning *et al.*, 2019; Jurburg *et al.*, 2019; Glick *et al.*, 1978; Liao *et al.*, 2012). This increase was observed in Chapter 4 but only in Trial Two. In Trial One, no colonisation by *Candidatus* Arthromitus was detected by quantitative PCR at any time point. In both experiments described in Chapter 3, treated chicks were colonised by *Candidatus* Arthromitus earlier than control chicks. As in the case of Ruminococcacaeae, it's likely that *Candidatus* Arthromitus spores were present in the transplant material. Therefore, the delay in colonisation until 7 d.p.h could be attributed to suboptimal conditions in the ileum for spore germination or vegetative cell growth rather than a lack of exposure. However, the difference in *Candidatus* Arthromitus colonisation between treated and control chicks could still be explained by delayed exposure rather than presence of other bacteria changing ileal conditions since the abundance of environmental *Candidatus* Arthromitus spores able to colonise the control chicks was not known.

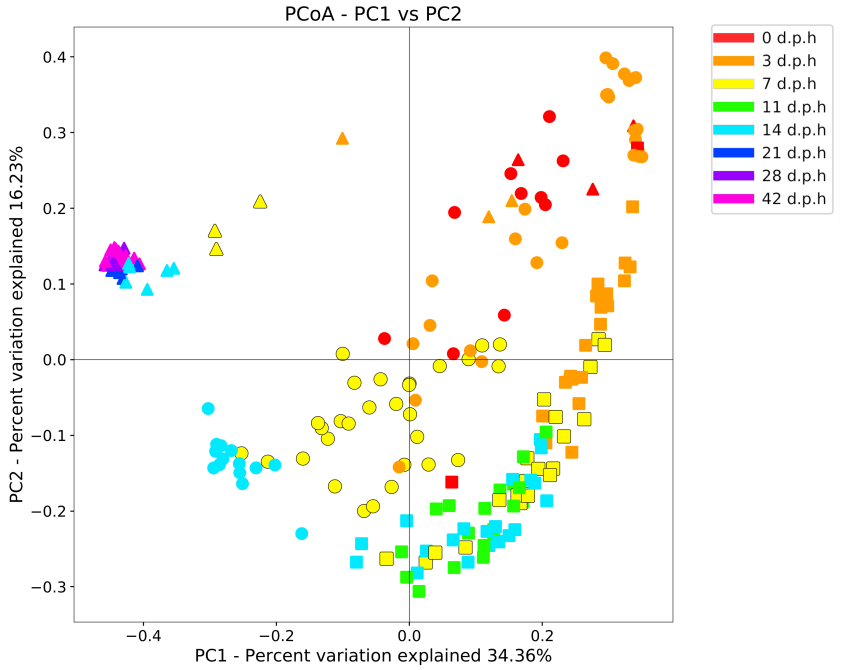
Other bacterial taxa associated with colonisation at later time points included *Romboutsia* and *Turicibacter* which colonised the ileum around 14 d.p.h. In this case it is difficult to discern whether their establishment in the ileum was linked to the prior presence of other taxa as in the case of Lachnospiraceae and Ruminococcacaee. A pattern of later colonisation by these genera has been observed in other studies (Jurburg *et al.*, 2019; Glendinning *et al.*, 2019). Jurburg *et al.* (2019) suggest that their later colonisation can be attributed to slow growth. However, in Chapter 3, presence of *Romboutsia* and *Turicibacter* was noted in the ilea of treated chicks at 0 d.p.h. If the absence of these taxa from the ileum until later time points were due to growth rates, this initial population could be expected to persist in the ileum albeit at a lower relative abundance. Instead, these taxa were no longer present in the ilea of treated chicks at 3 d.p.h, returning only at 14 d.p.h. This provides evidence that

some host or environmental factor in the ileum acts against colonisation by *Romboutsia* and *Turicibacter* until later time points.

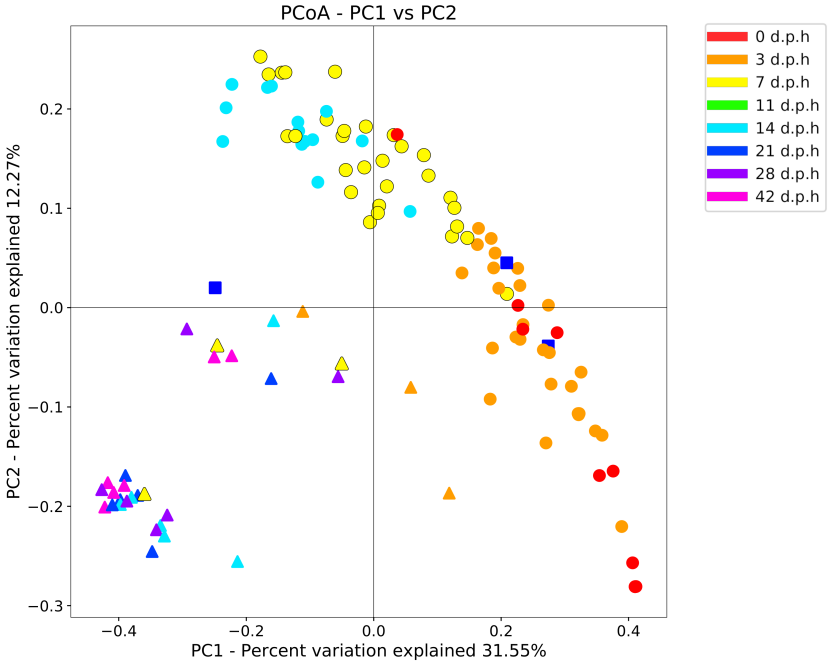
5.2 Environmental Exposure Was The Largest Factor in Determining Microbiome Composition

As mentioned previously, samples from Chapter 2 contained ASVs assigned to taxa such as Bifidobacteriaceae, Burkholderiaceae and Bacteroidaceae which were not observed in other experiments. Differences in microbiome composition between experiments are illustrated in Figure 5.1 where the unweighted UniFrac distance between samples is displayed. While there is visible clustering of samples by experiment and age, there is greater similarity between samples from Chapters 3 and 4 with samples from Chapter 2 clustering further away. Differences in caecal microbiome composition between the three experiments described in this thesis can also be viewed at the ASV level in Figure 5.2. This figure demonstrates that the ASV profile present in caecal samples between different experiments was consistently different. Samples from Chapter 3 were an intermediate between samples from Chapters 2 and 4, unsurprisingly since ASVs from Chapter 2 would have been present in the transplant material used.

Some differences in intestinal microbiome between experiments could be explained by the different provenance of the chicks. Chicks used in the experiments detailed in Chapters 3 and 4 were bought from a hatchery within an hour's drive of the experimental site. They were collected in the same vehicle by the same person and delivered directly to the experimental housing. In contrast, chicks used in the experiment described in Chapter 2 were bought from a hatchery with a journey of approximately 5 hours to the experimental site. A different vehicle was used to collect the chicks with two people present during the journey. This difference in environmental exposure could have allowed colonisation by a wider range of bacterial taxa. However, the relative abundance of these taxa did not increase until 3 or 7 d.p.h. This delayed increase in relative abundance does not preclude that the initial colonisation occurred on the journey from the hatchery to the experimental



(a) Caecum



(b) Ileum

Figure 5.1: Beta diversity between caecal and ileal samples from all experiments

Sample metadata is identified by age (colour) and experiment (Chapter 2: ▲ ; Chapter 3: ● ; Chapter 4: ■).

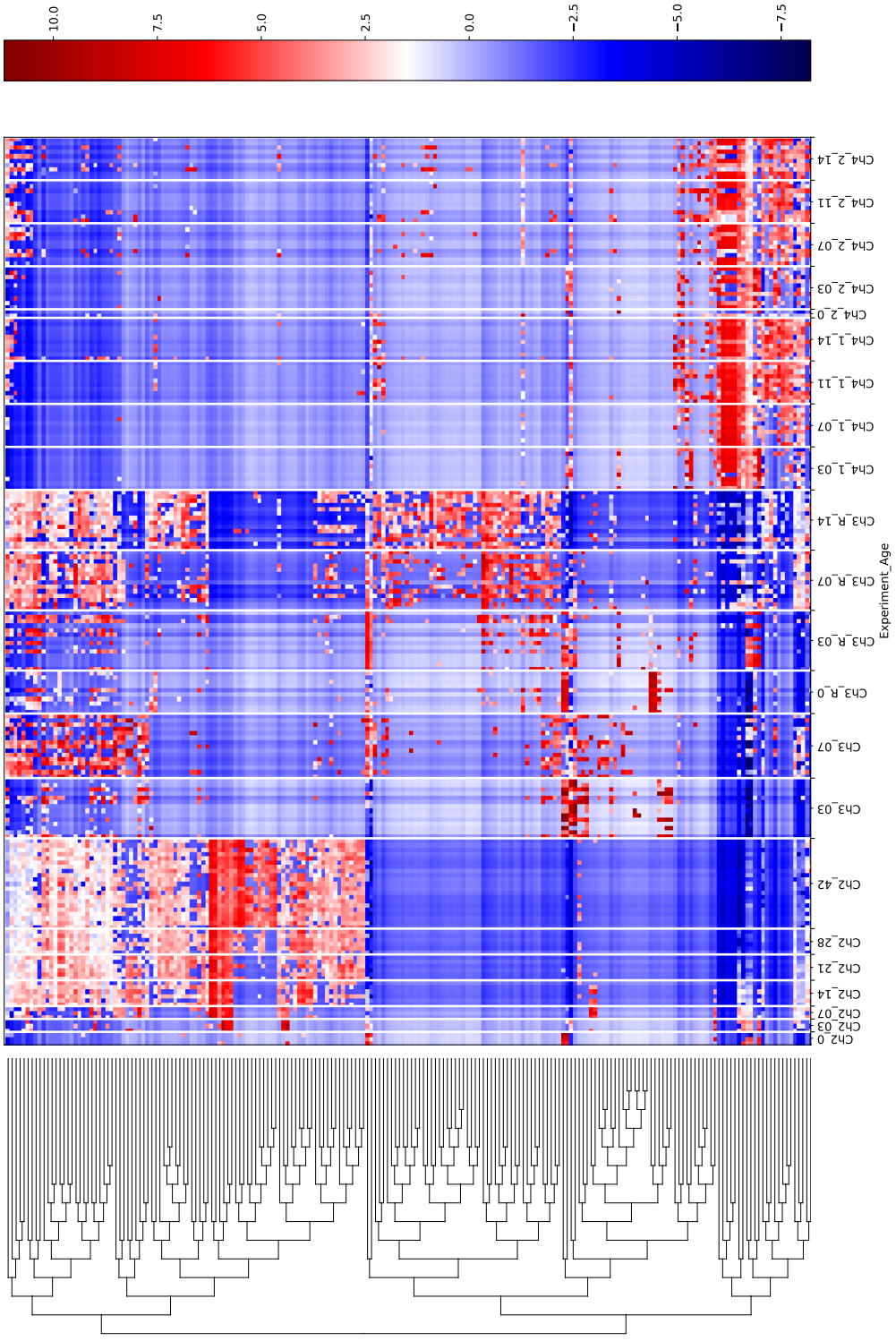


Figure 5.2: ASV log abundance in caecal samples from all experiments
Samples are arranged by experiment (Ch2: Chapter 2; Ch3: Chapter 3 - Pilot Experiment; Ch3_R: Chapter 3 - Repeat Experiment; Ch4_1: Chapter 4 - Trial One; Ch4_2: Chapter 4 - Trial Two) and by age in days post hatch.

housing. Since only a small number of chicks were sampled it's possible that one or two unsampled chicks harboured Bacteroidaceae or Burkholderiaceae which were able to persist in low numbers until optimal caecal conditions allowed expansion of the population and spread to other members of the flock. Additionally, pooling of samples may have obscured the presence of low abundance taxa.

Differences in microbiome composition between studies conducted under similarly biosecure conditions have been previously described. Stanley *et al.* (2013*a*) reported significant differences in microbiome composition between three trials conducted over a five month period despite chicks being obtained from the same hatchery and housed in the same biosecure housing. However, research examining the origin of bacteria colonising the chicken intestinal tract has not been conducted. The contribution of different environmental bacteria to the development of the chicken intestinal microbiome is an important consideration for the design of measures to influence its development. If, for example, bacteria colonising the intestine at the hatchery and during transfer to rearing units contribute more to the development of the intestinal microbiome than bacteria which are present on farm then the timing of interventions to influence the microbiome will be fixed at hatch. Alternatively if each broiler unit is found to have a unique microbiome which is defined by bacterial colonisation during the rearing period then on-farm interventions will be more successful.

5.3 Topical Application of a Mixed Bacterial Community to Eggs Was More Successful in Altering Microbiome Composition Than an Oral Multistrain Probiotic

The results discussed in Chapter 3 showed a significant effect of topical application of adult caecal contents to eggs during incubation. The effects were most visible at 3 d.p.h with accelerated development of the caecal microbiome with some features of the ileal microbiome also affected. This accelerated development was associated with earlier appearance of taxa associated with a mature intestinal microbiome and reduced abundance of potential

pathogens. In contrast, an oral multistrain probiotic containing *Bacillus amyloliquefaciens* (BA) had no effect on intestinal microbiome development or the abundance of potential pathogens. Additionally, ASVs from the transplant material were recovered from treated chicks while there was no evidence of BA colonisation of the caecum or ileum.

One factor in this observed difference between experimental outcomes was the multispecies nature of the transplant material compared to the multistrain, but monospecies, probiotic. Commericially available in-feed probiotics are often composed of a single species although others are a mixture of up to three species. In general, the taxonomy of these probiotics is limited to members of *Lactobacillus*, *Bacillus*, *Bifidobacterium* or *Enterococcus* (Gadde *et al.*, 2017). Direct comparisons between administration of multispecies and multistrain or monospecies probiotics in poultry are scarse. Two studies examined the effect of either single species or multispecies *Lactobacillus* administration on a variety of parameters including *Escherichia coli* colonisation and volatile fatty acid production. Both studies concluded that while treated chicks had reduced *E. coli* colonisation and increased fatty acid production compared to untreated controls, there were no significant differences between mono- and multispecies treatments although there was a lower mortality in multispecies treated chicks (Jin *et al.*, 1998, 2000). It has subsequently been suggested that this lack of effect could have been caused by the method used to produce the multispecies probiotic since *Lactobacillus* cultures were fermented together before administration to chicks. As a result, the final composition of the multispecies probiotic was not known and could have been dominated by one or two *Lactobacillus* species (Timmerman *et al.*, 2004). Reviews of comparisons between mono- and multispecies probiotics in other species have concluded that multispecies probiotics provide better results for a variety of parameters (Timmerman *et al.*, 2004; Chapman *et al.*, 2011). This is the case even when components of the multispecies mixture are used as the monospecies comparison (Chapman *et al.*, 2011). Although the reason for the superiority of multispecies probiotics is not known some theories have been proposed. The most likely is that provision of a variety of species results in a higher chance of successful colonisation by probiotic bacteria. Since intestinal conditions including pH and other resident microbiota will vary between invividuals, the “one size fits all” approach

of a monospecies probiotic is unlikely to be as successful as a multispecies product. Equally, it's possible that synergistic effects between components of multispecies probiotics could increase the chance of successful colonisation.

While successful colonisation by some ASVs in the transplant material and the failure of BA to colonise could be explained by chance and probability, other factors may have influenced this difference. The transplant material used in Chapter 3 was obtained directly from a mature chicken. While freezing will have reduced the viability of some vegetative cells, the provenance of the bacteria provides good support for their suitability to colonise the chicken gastrointestinal tract. In contrast, the BA strains used in Chapter 4 were not cultured directly from the intestinal contents of chickens. Instead two were cultured from turkey litter and the source of the third is unknown (EFSA FEEDAP Panel, 2016). To my knowledge, there is no evidence in publically available literature that these strains are able to successfully colonise the chicken gastrointestinal tract. While these results should not be taken as evidence that these BA strains are unable to germinate and colonise the chicken intestine it may indicate that local conditions inhibited colonisation and therefore the success of the intervention. Another factor that may have influenced colonsiation ability is generational time between original isolation and readministration to a host gastrointestinal tract. Survival in the gastrointestinal tract depends on a variety of genetic factors which are of no benefit to growth and survival under laboratory culture conditions. Presumably the BA strains used in Chapter 4 have undergone a number of rounds of laboratory and commercial culture since their original isolation. As a result, these strains may have lost genetic elements which would facilitate colonisation of the gastrointestinal tract but which are of no use for growth under laboratory or commercial conditions. Considering that bacteria in probiotic treatments will be competing with commensals which are adapted for intestinal colonisation any reduction in fitness will affect the success of a probiotic treatment.

5.4 The Future of Poultry Probiotics

5.4.1 The objective of probiotic administration should be rapid colonisation and succession by environmental bacteria

The addition of in-feed probiotics and prebiotics is widely practised in the poultry industry especially in the European Union in light of restrictions on antibiotics in feed. Consequently there has been an increase in publications exploring the effects of probiotics on a diverse range of outcomes including alteration of the intestinal microbiome (Li *et al.*, 2015; Zhang *et al.*, 2015; Baldwin *et al.*, 2018), increased disease resistance (Ritzi *et al.*, 2014; Willis and Reid, 2008) and better production parameters (Li *et al.*, 2015; Zhang *et al.*, 2015; Baldwin *et al.*, 2018). Indeed, research investigating the effect of in-feed probiotics on production, immunological or morphological parameters alongside changes in the intestinal microbiome often ascribe any positive effect on these parameters to an influence of the treatment. However, these are rarely examined in the context of normal succession and development of the intestinal microbiome. The composition of the intestinal microbiome varies significantly between experiments conducted under similar circumstances as illustrated in the experiments presented in this thesis and by other authors (Stanley *et al.*, 2013*b*). It is therefore reasonable to assume that there is huge variability in intestinal microbiome composition between commercial poultry units. As a result, if a probiotic product is shown to significantly alter certain taxa at 14, 28 or 42 days post hatch under controlled experimental conditions it could be questioned how applicable these results are to industry. If changes in the microbiome are linked to differences in other parameters such as performance and disease resistance then the positive effect of the probiotic may rely on the presence of those taxa in commercial units. However, if a probiotic treatment is shown to increase successful colonisation of the gastrointestinal tract by environmental bacteria as evidenced by a more rapid succession as outlined above it could be argued that such an outcome would be more applicable to industry since it does not rely on the presence or absence of specific taxa. The best way to ensure pathogen resistance across multiple environments

with different microbiome compositions is to ensure rapid succession and development of the intestinal microbiome. This is supported by the observation that early colonisation by taxa such as *Candidatus* Arthromitus, *Bacteroides* and *Eggerthella* is positively correlated with performance whereas later colonisation by the same taxa is negatively correlated with performance suggesting that delayed microbiome colonisation has a detrimental effect on production (Johnson *et al.*, 2018). While specific taxa may play a role in treatment success, timing of treatment is likely to have a greater role. As such, probiotic investigations should focus on speed of succession as a parameter rather than comparing taxonomic composition between experimental groups. This may include comparisons such as first colonisation by Lachnospiraceae, Ruminococcaceae and Bacteroidaceae in the caecum and *Candidatus* Arthromitus, *Romboutsia* and *Turicibacter* in the ileum. Further research regarding the successional development of the intestinal microbiome in poultry is likely to reveal other standard biomarkers which would be useful benchmarks for measuring the effect of probiotics on the microbiome.

5.4.2 Probiotic candidates should be identified from the normal microbiome

As previously discussed, commercially available probiotics are limited to a small selection of bacterial genera including *Bifidobacterium* and *Lactobacillus* (Gadde *et al.*, 2017). While *Lactobacillus* has been shown to be a consistent and major contributor to the ileal microbiome it is not highly abundant in the caecal microbiome. Other taxa such as *Bifidobacterium* and *Bacillus* are present in the caecal and ileal microbiomes but are rarely considered major constituents. The use of other commensals as probiotics should be considered to create a new generation of probiotic products.

Potential candidates for development as probiotics

From the experiments presented in this thesis and the literature some taxonomic groups should be considered likely targets for probiotic development

Lachnospiraceae formed a major component of the early caecal microbiome and, as

discussed previously, were pioneer colonisers whose presence appeared to facilitate succession of other taxa. As such, members of this taxonomic group should be considered candidates for future probiotics. The results presented in Chapter 3 demonstrate that Lachnospiraceae can be readily transferred to recipient chicks via an aerosol application of spores to the eggshell during incubation. This provides a convenient and non-invasive intervention which could improve microbiome development in chicks. Lachnospiraceae is a diverse family of bacteria and screening of cultured strains should be conducted to assess suitability as probiotics.

Ruminococcaceae were also present in the caecal microbiome although at later time points compared to Lachnospiraceae. The results presented in Chapter 3 showed that despite this pattern of later colonisation, application of Ruminococcaceae spores to the eggshell during incubation resulted in increased abundance of this taxon. Consequently, members of Ruminococcaceae could be considered for future probiotics within the same treatment as Lachnospiraceae. Members of Ruminococcaceae such as *Faecalibacterium prausnitzii* have been proposed as potential probiotics and have shown promise in other species (Carlsson *et al.*, 2013; Sokol *et al.*, 2008). Other genera within Ruminococcaceae should be explored for probiotic potential as well.

Bacteroidaceae was a large constituent of the caecal microbiome in Chapter 2. Subsequent failure to colonise chicks in Chapters 3 and 4 was likely due to Bacteroidaceae's inability to form spores and survive more than 24 hours in an oxygen rich environment (Medvecky *et al.*, 2018). However, Bacteroidaceae is often cited as an important member of the caecal microbiome since an earlier presence is correlated with improved performance (Johnson *et al.*, 2018). Despite the failure to transfer Bacteroidaceae in Chapter 3, this taxon should not be discounted as a probiotic candidate since exposure in the 24 hours immediately post hatch results in successful colonisation by 7 d.p.h (Kubasova *et al.*, 2019). As a result, the application strategy of Bacteroidaceae would be different for spore-formers such as Lachnospiraceae and Ruminococcaceae with application around hatch likely to result in increased transfer to recipient chicks.

***Candidatus* Arthromitus** were an important signal of ileal microbiome development.

Due to the importance of *Candidatus* Arthromitus in immune development and evidence showing that early colonisation is correlated with improved performance (Johnson *et al.*, 2018), this taxon should be considered as a potential probiotic candidate. In Chapter 3, earlier *Candidatus* Arthromitus colonisation was observed in chicks exposed to adult caecal contents. This may have been due to presence of spores in the caecal content which, like Ruminococcaceae, were able to persist in the gastrointestinal tract until optimum conditions allowed for colonisation. Equally, the presence of other taxa within the microbiome may have allowed earlier colonisation by environmental *Candidatus* Arthromitus. *Candidatus* Arthromitus presents a unique challenge as its epithelial-associated life cycle would be difficult to replicate industrially, however, *in vitro* culture techniques have been described (Schnupf *et al.*, 2015).

Romboutsia and ***Turicibacter*** formed large parts of the mature ileal microbiome in Chapter 2 and were also present in the ileal microbiome from 14 d.p.h in Chapter 3. Currently, most commercially available probiotics targeted at the ileum are composed of *Lactobacillus* or *Enterococcus*. While these taxa do form the majority of the ileal microbiome at early time points their supplementation should not come at the expense of other taxa such as *Romboutsia* or *Turicibacter*. Just as *Lactobacillus* contributes to host metabolism, there is evidence to show that these less researched taxa also occupy a specific metabolic niche in the ileal microbiome and therefore supplementation via probiotics may be beneficial. In the case of *Turicibacter* recent work has shown that the relative abundance of *Turicibacter sanguinis* in the mouse intestinal microbiome is positively influenced by levels of host serotonin. Additionally, an increased relative abundance of *T. sanguinis* resulted in changes to host lipid and steroid metabolism as evidenced by altered intestinal transcriptomes and lipidomes (Fung *et al.*, 2019). This link between host serotonin and the intestinal microbiome may be of particular interest in terms of damaging behaviours such as feather pecking, in which differences in both peripheral serotonin levels and the intestinal microbiome have been demonstrated between high and low-feather pecking chickens (van der Eijk *et al.*, 2019*a*,*b*). However, it should be noted that correlations between *Turicibacter* and inflammatory bowel disease have been found in mice. As such, further research would

be required to determine the influence of this genus on host physiology, metabolism and disease (Fung *et al.*, 2019). *Romboutsia* remains a poorly characterised genus due to its relatively recent isolation in the laboratory (Gerritsen *et al.*, 2017). Despite this lack of knowledge regarding the genus, its presence at a high relative abundance in the mature ileal microbiome should signal it as a genus of interest in the context of probiotics.

The benefits of commensals as probiotics

Currently, the provenance of probiotic species varies. While some are cultured from chicken faeces or intestinal contents (Kizerwetter-Świda and Binek, 2016) other commercially available probiotics, such as those used in Chapter 4, are cultured from environmental sources such as litter or soil (Lee *et al.*, 2008). It has been suggested that the use of bacteria cultured from sources other than the gastrointesintal tract of the target species is irrelevant since probiotics exert their effect by producing factors which facilitate colonisation by commensals (Wu *et al.*, 2011). While this may apply when live cultures of bacteria are given along with supernatant, many commercial probiotics are administered in feed and must therefore colonise the gastrointestinal tract or germinate within the intestine in order to produce factors which would subsequently aid commensal colonisation. Additionally, others have suggested that probiotics aid in pathogen exclusion by producing antimicrobial factors. The success of pathogen exclusion by this method is likely to depend on a probiotic bacterium's ability to successfully colonise the host intestine. In the case of the probiotic used in Chapter 4 the absence of any effect on microbiome development was accompanied by no evidence of intestinal colonisation by the probiotic. In contrast, commensals recently derived from a host gastrointestinal tract are uniquely adapted to colonise and thrive in that environment. As a result, any beneficial effect due to their presence is more likely to occur and persist. This was demonstrated in Chapter 3 where 20% of topically applied taxa were recovered from treated chicks in the repeat experiment.

Another advantage of using commensals as opposed to environmental bacteria as probiotics is an increased likelihood of a positive beneficial effect. Commensals have co-evolved with their hosts and as a result are more likely to exert some effect on the host, be

that by producing beneficial metabolites or via direct interactions with the host immune system. *Candidatus* Arthromitus provides an example of such a symbiotic relationship. *Candidatus* Arthromitus' life cycle is so closely associated with the intestinal epithelium of the host that *Candidatus* Arthromitus species have developed host specificity. The host's recompense for this close association comes in the form of immune stimulation which ultimately benefits the host. In contrast, other bacteria associating so closely with intestinal epithelial cells would normally be associated with pathology. As well as co-evolving with their host, commensals have co-evolved with each other. As a result, the likelihood of metabolic or other relationships exisiting with other commensal bacteria is greater for a commensal rather than environmentally derived probiotic. These interactions between commensals need not be cooperative, indeed, an over-reliance on cooperative interactions in the microbiome can lead to a less stable community (Coyte *et al.*, 2015).

The fact that many commensal bacteria of the chicken intestinal tract are spore-forming presents another benefit to their use as probiotics. Spores are resistant to a wide variety of environmental conditions and remain viable for long periods of time. As a result, they are suitable for inclusion in animal feeds which are often processed at high temperatures and pressures. Additionally, we can be more certain that spores derived from commensals will successfully germinate and colonise the gastrointestinal tract. There is conflicting evidence whether spores of probiotic *Bacillus* strains are able to germinate in the gastrointestinal tract (Spinosa *et al.*, 2000; Casula and Cutting, 2002; Cartman *et al.*, 2008).

Challenges that need to be overcome

While there would be benefits to deriving probiotics from commensal species some challenges will need to be overcome. The principal obstacle is the difficulty of culturing many commensal bacteria under laboratory, and subsequently industrial, conditions. One of the attractions of *Lactobacillus*, *Bacillus* and other commerical probiotics is the relative ease with which they can be cultured in captivity. In contrast, commensals such as Lachnospiraceae and Ruminococcaceae are so difficult to culture that the majority of species are known only from 16S rRNA gene sequences recovered from environmental samples. This will have to be

overcome if a new generation of probiotics is to be developed. However, new culture media and techniques are being developed which have resulted in the isolation of new species belonging to poorly characterised taxa from both the human and chicken gastrointestinal tract (Browne *et al.*, 2016; Medvecky *et al.*, 2018). These new laboratory techniques could be applied to industry or expanded and modified to culture a greater diversity of previously unidentified bacteria.

Spore-formers were mentioned in the previous section as a benefit to selecting commensal bacteria for use as probiotics. However, a proportion of the microbiome do not rely on spores to transfer between hosts instead relying on microaerotolerance (Medvecky *et al.*, 2018). While these taxa can survive exposure to oxygen for up to 24 hours, this limits their ability to be used as in-feed probiotics. Consequently, other delivery methods would need to be used or treatments could focus on indirectly increasing colonisation by these species.

5.4.3 Single or multispecies mixtures

Commercially available probiotics usually consist of only one species or multiple strains of the same species although competitive exclusion products are available that contain multiple species. The results presented in this thesis and available in the literature suggest that the current focus on single species probiotics should be changed to the development of multispecies products. Reviews of experiments comparing single and multispecies probiotics have found that multispecies products often provide better outcomes than single species probiotics (Chapman *et al.*, 2011; Timmerman *et al.*, 2004). A review has also found that in experiments in which multispecies probiotics are compared to the effect of administering each constituent separately, the multispecies product resulted in improved performance in 75% of cases (Chapman *et al.*, 2011). The studies included in reviews were conducted to compare the effects of probiotics on reducing intestinal colonisation by pathogenic bacteria. However, the conclusion that a multispecies product would result in improved microbiome development and composition is supported by the results presented in Chapters 3 and 4 in which topical treatment of eggs with a mixture of caecal commensals resulted in accelerated microbiome development whereas an oral multistrain probiotic did not. This is not a

surprising result and it would be interesting to conduct a similar experiment using a defined, cultured mixture of commensal bacteria as a topical egg treatment rather than diluted adult caecal contents. Previously, the logic behind single species probiotics was that the probiotic bacteria would facilitate and improve commensal colonisation resulting in benefits for the host such as increased concentrations of butyrate producing bacteria. However, it is likely to be more efficient and reliable to directly administer spores or vegetative cells of commensal bacteria to achieve the same result.

One particularly important aspect of developing multispecies probiotics for poultry is the vastly different composition of the ileal and caecal microbiomes. As the results presented in Chapter 2 show, there are few taxa shared between the caecal and the ileal microbiomes especially in their mature state. These differences may occur at the species level as shown by some Lactobacillaceae species that were found to be more abundant in the caecum despite the prevalence of other Lactobacillaceae species in the ileum. Consequently, it cannot be expected that a single species or multistrain probiotic would result in effects on microbiome composition in both organs.

5.4.4 Administration of probiotics to poultry

Oral delivery of probiotics in feed or water is currently the most common delivery system used in the poultry industry. However, the results of Chapter 3 show that an aerosol application would also be a viable delivery system. The implementation of such a system raises some questions. It is now widely accepted that probiotic administration is more effective when delivered as early as possible. This would suggest that the optimum time for application would be in the hatchery, either to the eggshell before hatch or directly to the chicks post-hatch. The implications of probiotic treatment in the hatchery need to be explored more fully. Any effective probiotic treatment in the hatchery would influence future microbome composition as short exposures have been shown to influence microbiome development in both Chapter 3 and the wider literature (Ballou *et al.*, 2016; Kubasova *et al.*, 2019). It is not currently clear whether colonisation by environmental bacteria on farm or by a defined mixture at hatch results in a more stable adult microbiome which

is able to resist insults of exposure to pathogens and physiological stress. It could be argued that each farm's environmental microbiome has already been selected for under the conditions present on that farm and therefore are more likely to persist in the chickens compared to a mixture applied in the hatchery. Additionally, treatment at the hatchery might limit the choice of probiotic mixtures that farmers have. This would depend on the implementation but delivery systems would need to be cleaned thoroughly between batches if different probiotic treatments were required. The resulting increased work and administration required may dissuade hatcheries from offering a variety of probiotic products depending on customer preference and instead implement a standard probiotic mixture for all chicks. Even if farmers were happy to accept a standard probiotic treatment there may be another disadvantage depending on the success and persistence of the treatment. If probiotic treatment in the hatchery resulted in persistence of administered taxa in the microbiome there is a risk of increasing homogeneity between farm microbiomes. Since the microbiome is linked to disease resistance it seems unwise to reduce wider microbiome diversity. In other contexts, reduced diversity often leads to increased susceptibility to developing pathogens and it should be considered whether the same would be true of the intestinal microbiome.

An alternative would be to treat chicks on arrival at each farm with a customised mixture of commensals derived from healthy adult chickens raised in the same location and under similar conditions. This would more closely mimic the conditions of the experiment presented in Chapter 3. The treatment presented in Chapter 3 resulted in the earlier appearance of later colonisers such as Ruminoccocaceae and Clostridiales vadin BB60 compared to controls. A factor in this may have been that chicks remained in the same room after hatch allowing transplanted bacteria to persist and build up in the environment. This is more analogous to treatment on farm where chicks would be treated before entering the rearing shed where they would remain for the entirety of the production period. Chicks are constantly moved and stressed for the first 24 to 72 hours post-hatch which may result in reduced efficacy and persistence of any bacteria delivered in a probiotic treatment by the hatchery. Another possible benefit of on-farm probiotic application is the potential for

developing staged probiotics whereby early colonisers are administered on arrival with later colonisers provided in a second or third treatment at specific control points. This would provide a higher dose of later colonisers like Ruminococcaceae, *Candidatus* Arthromitus and *Romboutsia* at the optimal time rather than relying on persistence of these taxa within the chickens or their environment. However, treatment on farm does not follow the rule that earlier treatment is optimal. The delay in treatment from hatch to arrival on farm may allow potential pathogens present in the hatchery and wider environment to establish a stable population within the gastrointestinal tract.

5.5 Future Experiments

While the experiments presented in this thesis endeavoured to shed light on the development of the intestinal microbiome in chickens and interventions to influence bacterial colonisation and succession, the results and discussion have highlighted several areas of interest which merit further development.

Firstly, although the results presented in this thesis established an approximate successional order in both the caecum and ileum, further research to investigate the host and environmental factors which influence bacterial colonisation should be conducted. *Candidatus* Arthromitus presents a good target for initial investigations as colonisation usually occurs within a well-defined time period. In this case, host factors such as IgA levels or expression of suitable binding sites for *Candidatus* Arthromitus on epithelial cells could be explored to define a temporal limit to ileal colonisation by this genus.

Additionally, further work to determine the interdependency of bacterial taxa is required. While this thesis postulates that prior colonisation by Lachnospiraceae facilitated caecal colonisation by Ruminoccaceae this hypothesis needs to be confirmed under more strictly controlled conditions at different experimental sites. *In vitro* experiments to determine a mechanism by which Lachnospiraceae might create a favourable environment for Ruminococcaceae would add further depth to the understanding of caecal microbiome development.

Observing the spread of commensal bacteria through a group of chicks would allow further insights into how commensals are able to persist within a flock either having been transferred to a few chicks during transport or picked up from the environment. Such an investigation would also allow for better targeting of transplant treatments. It's possible that only a subset of chicks need to be treated with aerosolised probiotics for effective transfer to all members of the group. This would allow lower doses to be administered and create time efficiencies.

As well as determining the effects of other bacterial taxa on colonisation, host factors which influence bacterial colonisation should be further investigated. In particular, the identification of host genomic characteristics which favour the colonisation by beneficial taxa could be the target of future research. This may include reviewing genes associated with enteric receptors or the host immune response which modulate the composition of the intestinal microbiome. While host genome is an important factor when considering resistance to pathogens, current research considering how broiler chicken genomics influences the development and maintenance of the intestinal microbiome is limited to observational studies with little mechanistic insight (Lumpkins *et al.*, 2010; Schokker *et al.*, 2015; Zhao *et al.*, 2013). In this respect, the importance of using a "multi-omic" approach to examine how host genome, host and bacterial gene expression, metabolomic profile and the presence of other microorganisms (viruses and fungi) influence microbiome colonisation and dynamics should be considered as a useful future approach for determining mechanisms within biological systems which can inform and target interventions.

While the theoretical and mechanistic aspects of microbiome-host interactions should be the focus of more research, this should not come at the expense of continuing to observe the outcome of interventions and on production parameters which will ultimately decide their suitability for field use. The studies included in this thesis lacked the ability to correlate interventions to improved body weight gain or feed conversion ratios, the most common benchmarks used to judge success in the broiler industry. Future studies should be designed to take into account production parameters to satisfy the need to demonstrate to producers that uptake of interventions to improve microbiome colonisation or composition are in their

interest.

In the laboratory, further work must be carried out to culture and characterise intestinal commensals. In particular members of Lachnospiraceae, Ruminococcaceae, *Candidatus* Arthromitus, *Romboutsia* and *Turicibacter* should be considered suitable targets. The techniques used by other research groups for collection, treatment and culture provide a good starting point (Browne *et al.*, 2016; Medvecky *et al.*, 2018). In particular, encouraging germination of spore forming bacteria by addition of sterile rumen fluid to culture media could be extended to using sterile caecal content supernatant which may contain specific molecular signals to stimulate germination of commensal spores. As well as classifying and genome sequencing of new species, determining a culture technique which maximises recovery of commensal bacteria from samples would allow for customised probiotic mixes to be created on a farm-by-farm basis.

Finally, experiments are required to determine optimal conditions for probiotic administration. As discussed above, it is currently unknown whether a predefined mixture of probiotic bacteria applied in the hatchery would be able to successfully influence the composition of the intestinal microbiome under commercial conditions during which chicks are often transported long distances and physiologically stressed. Subsequently, any probiotic bacteria administered in the hatchery may not be sufficient to withstand colonisation by environmental bacteria which may have been selected for due to on-farm conditions. However, delaying administration until arrival on farm may leave a window of opportunity for colonisation by potential pathogens and reduce the effect of the probiotic. As such application of probiotic mixtures in hatcheries under close to commercial conditions should be compared to on-farm administration in older chicks. Additionally, the benefits of staged probiotic mixture administration could be investigated to determine whether it is more effective than an “all-in-one” application at hatch.

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Appendices

Appendix A

Appendix - The Development of the Chicken Intestinal Microbiome

Ingredients		Additives (per kg)	
	Vitamins	**Trace Elements**	**Antioxidants**
Starter	E672 Vitamin A 11250 iu E671 Vitamin D3 3000 iu	E1 Iron (Ferous Sulphate Monohydrate) 10 mg, E2 Iodine (Calcium Iodate Anhydrous) 1016 µg, E3 Cobalt (Cobaltous Acetate Tetrahydrate) 287.5 µg, E4 Copper (Cupric Sulphate Pentahydrate) 5 mg, E5 Manganese (Manganous Oxide) 80 mg, E6 Zinc (Zinc Oxide) 60 mg, E8 Selenium (Sodium Selenite) 150 µg	E320 Butylated hydroxyanisole (BHA) 2 mg, E231 Butylated hydroxytoluene (BHT) 10 mg, E324 Ethoxyquin 40 mg, E310 Propyl gallate 2 mg
Grower	E672 Vitamin A 10280 iu E671 Vitamin D3 3080 iu	E1 Iron (Ferous Sulphate Monohydrate) 10 mg, E2 Iodine (Calcium Iodate Anhydrous) 1016 µg, E3 Cobalt (Cobaltous Acetate Tetrahydrate) 287.5 µg, E4 Copper (Cupric Sulphate Pentahydrate) 5 mg, E5 Manganese (Manganous Oxide) 80 mg, E6 Zinc (Zinc Oxide) 60 mg, E8 Selenium (Sodium Selenite) 150 µg	E320 Butylated hydroxyanisole (BHA) 2 mg, E231 Butylated hydroxytoluene (BHT) 10 mg, E324 Ethoxyquin 40 mg, E310 Propyl gallate 2 mg

Table A.1: A list of ingredients in starter and grower diets

Taxonomy	y0		y2		y6		y13		y28		y48		y73		Total
	Num	Denom	Num	Denom	Num	Denom	Num	Denom	Num	Denom	Num	Denom	Num	Denom	
Ruminococcaceae	126	39	126	0	118	8	8	0	8	0	6	2	1	5	447
Lachnospiraceae	73	49	73	0	60	13	13	0	12	1	8	4	2	6	314
Clostridiaceae 1	23	0	21	2	0	21	21	0	20	1	19	1	18	1	148
Enterococcaceae	23	0	20	3	0	20	17	3	17	0	16	1	15	1	136
Enterobacteriaceae	19	0	19	0	2	17	17	0	15	2	2	13	2	0	108
Clostridiales vadinBB60 group	26	3	26	0	25	1	1	0	1	0	1	0	1	0	85
Peptostreptococcaceae	12	0	11	1	0	11	11	0	4	7	4	0	4	0	65
Lactobacillaceae	10	0	8	2	2	6	4	2	1	3	1	0	1	0	40
Christensenellaceae	9	1	9	0	9	0	0	0	0	0	0	0	0	0	28
Eggerthellaceae	5	0	5	0	2	3	3	0	3	0	3	0	0	3	27
Erysipelotrichaceae	6	0	4	2	2	2	2	0	2	0	2	0	0	2	24
Coriobacteriaceae	3	0	3	0	1	2	2	0	2	0	2	0	0	2	17
Bacillaceae	4	1	4	0	4	0	0	0	0	0	0	0	0	0	13
Peptococcaceae	4	0	4	0	4	0	0	0	0	0	0	0	0	0	12
Aerococcaceae	2	0	2	0	0	2	2	0	1	1	1	0	1	0	12
Corynebacteriaceae	2	0	2	0	0	2	2	0	1	1	1	0	1	0	12
Staphylococcaceae	2	0	2	0	0	2	2	0	0	2	0	0	0	0	10
Eubacteriaceae	2	0	2	0	1	1	1	0	1	0	1	0	0	1	10
Streptococcaceae	1	0	1	0	0	1	1	0	1	0	1	0	1	0	7
Dermabacteraceae	1	0	1	0	0	1	1	0	1	0	1	0	1	0	7
Clostridiales	2	0	2	0	2	0	0	0	0	0	0	0	0	0	6
Family XIII	2	0	2	0	2	0	0	0	0	0	0	0	0	0	6
Burkholderiaceae	1	3	1	0	1	0	0	0	0	0	0	0	0	0	6
Bacteroidaceae	1	2	1	0	1	0	0	0	0	0	0	0	0	0	5
Planococcaceae	1	0	1	0	0	1	1	0	0	1	0	0	0	0	5
Atopobiaceae	1	1	1	0	1	0	0	0	0	0	0	0	0	0	4
uncultured rumen bacterium	1	1	1	0	1	0	0	0	0	0	0	0	0	0	4
Mollicutes RF39	1	0	1	0	1	0	0	0	0	0	0	0	0	0	3
Defluviitaleaceae	1	0	1	0	1	0	0	0	0	0	0	0	0	0	3
gut metagenome	1	0	1	0	1	0	0	0	0	0	0	0	0	0	3
Firmicutes bacterium CAG:822	1	0	1	0	1	0	0	0	0	0	0	0	0	0	3
Bifidobacteriaceae	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1

Table A.2: Taxonomy summary at the family level of ASVs in balances that were significantly different between caecal and ileal samples

Numerator (Num); Denominator (Denom)

Taxonomy	y0		y1		y2		y6		y10		y11		y18		Total
	Num	Denom	Num	Denom	Num	Denom	Num	Denom	Num	Denom	Num	Denom	Num	Denom	
Lachnospiraceae	54	0	0	0	53	1	52	1	1	0	14	38	14	0	228
Ruminococcaceae	40	0	0	0	40	0	40	0	0	0	12	28	12	0	172
Enterococcaceae	23	0	0	0	10	13	9	1	1	0	9	0	9	0	75
Enterobacteriaceae	16	0	0	0	14	2	10	4	4	0	10	0	10	0	70
Peptostreptococcaceae	11	1	1	0	11	0	7	4	1	3	6	1	4	2	52
Clostridiaceae 1	9	0	0	0	8	1	6	2	2	0	6	0	4	2	40
Lactobacillaceae	8	2	2	0	6	2	4	2	2	0	2	2	2	0	34
Corynebacteriaceae	4	0	0	0	4	0	3	1	0	1	3	0	3	0	19
Staphylococcaceae	4	0	0	0	4	0	2	2	0	2	2	0	2	0	18
Peptococcaceae	3	0	0	0	3	0	3	0	0	0	0	3	0	0	12
Dermabacteraceae	2	0	0	0	2	0	2	0	0	0	2	0	2	0	10
Christensenellaceae	2	0	0	0	2	0	2	0	0	0	2	0	2	0	10
Campylobacteraceae	2	0	0	0	2	0	2	0	0	0	1	1	1	0	9
Burkholderiaceae	2	0	0	0	2	0	2	0	0	0	1	1	1	0	9
Aerococcaceae	2	0	0	0	2	0	1	1	1	0	1	0	1	0	9
Bacteroidaceae	2	0	0	0	2	0	2	0	0	0	0	2	0	0	8
Erysipelotrichaceae	1	2	0	2	1	0	1	0	0	0	0	1	0	0	8
Bacillaceae	2	0	0	0	1	1	1	0	0	0	0	1	0	0	6
Streptococcaceae	2	0	0	0	1	1	1	0	0	0	0	1	0	0	6
Bifidobacteriaceae	1	0	0	0	1	0	1	0	0	0	1	0	1	0	5
Rhizobiaceae	1	0	0	0	1	0	1	0	0	0	1	0	1	0	5
Pseudomonadaceae	1	0	0	0	1	0	1	0	0	0	1	0	1	0	5
Paenibacillaceae	1	0	0	0	1	0	0	1	1	0	0	0	0	0	4
Coriobacteriaceae	1	0	0	0	1	0	1	0	0	0	0	1	0	0	4
Atopobiaceae	1	0	0	0	1	0	1	0	0	0	0	1	0	0	4
Planococcaceae	1	0	0	0	1	0	0	1	1	0	0	0	0	0	4

Table A.3: Taxonomy summary at the family level of ASVs in balances that were significantly different between time points in the ileum

Numerator (Num); Denominator (Denom)

Taxonomy	y2		y7		y8		Total
	Num	Denom	Num	Denom	Num	Denom	
Lachnospiraceae	35	18	9	0	26	0	88
Ruminococcaceae	17	23	9	0	8	0	57
Enterobacteriaceae	14	0	2	0	10	2	28
Peptostreptococcaceae	12	0	5	0	2	5	24
Enterococcaceae	9	0	5	0	0	4	18
Lactobacillaceae	8	1	3	2	3	0	17
Clostridiaceae 1	4	3	1	0	3	0	11
Corynebacteriaceae	4	0	3	1	0	0	8
Staphylococcaceae	4	0	2	2	0	0	8
Streptococcaceae	2	0	1	0	1	0	4
Dermabacteraceae	2	0	2	0	0	0	4
Aerococcaceae	2	0	1	0	1	0	4
Peptococcaceae	1	2	0	0	1	0	4
Burkholderiaceae	1	1	0	0	1	0	3
Campylobacteraceae	1	1	1	0	0	0	3
Atopobiaceae	1	0	0	0	1	0	2
Erysipelotrichaceae	1	0	0	0	1	0	2
Planococcaceae	1	0	0	0	1	0	2
Bifidobacteriaceae	1	0	0	0	1	0	2
Coriobacteriaceae	1	0	0	0	1	0	2
Paenibacillaceae	1	0	0	0	1	0	2
Bacteroidaceae	0	2	0	0	0	0	2
Christensenellaceae	0	2	0	0	0	0	2
Bacillaceae	0	1	0	0	0	0	1

Table A.4: Taxonomy summary at the family level of ASVs in balances that were significantly different ileal lumen and mucus

Numerator (Num); Denominator (Denom)

Taxonomy	y0		y1		y2		y3		y4		y5		y6		y7		y8		Total
	Num	Denom	Num	Denom	Num	Denom	Num	Denom	Num	Denom	Num	Denom	Num	Denom	Num	Denom	Num	Denom	
Ruminococcaceae	43	120	83	37	37	6	25	12	48	35	6	0	4	33	4	8	12	13	526
Lachnospiraceae	54	67	40	27	45	9	11	16	16	24	8	1	21	24	9	7	7	4	390
Clostridiales vadinBB60 group	1	28	25	3	1	0	3	0	17	8	0	0	0	1	0	0	2	1	90
Clostridiaceae 1	18	1	1	0	0	18	0	0	0	1	4	14	0	1	0	0	0	0	57
Enterobacteriaceae	17	0	0	0	2	15	0	0	0	0	3	12	2	0	0	0	0	0	51
Christensenellaceae	0	9	5	4	0	0	4	0	2	3	0	0	0	0	0	0	1	3	31
Enterococcaceae	9	0	0	0	0	9	0	0	0	0	6	3	0	0	0	0	0	0	27
Lactobacillaceae	3	4	2	2	1	2	0	2	2	0	2	0	1	0	0	2	0	0	23
Erysipelotrichaceae	2	4	3	1	0	2	1	0	1	2	2	0	0	0	0	0	0	1	19
Bacillaceae	0	5	4	1	0	0	1	0	0	4	0	0	0	0	0	0	1	0	16
Eggerthellaceae	3	2	2	0	0	3	0	0	0	2	3	0	0	0	0	0	0	0	15
Peptostreptococcaceae	3	1	0	1	1	2	0	1	0	0	1	1	1	0	0	1	0	0	13
Burkholderiaceae	3	1	0	1	3	0	0	1	0	0	0	0	3	0	0	1	0	0	13
Peptococcaceae	0	4	3	1	0	0	1	0	3	0	0	0	0	0	0	0	0	1	13
Bacteroidaceae	0	3	1	2	0	0	0	2	0	1	0	0	0	0	2	0	0	0	11
Coriobacteriaceae	2	1	1	0	0	2	0	0	1	0	2	0	0	0	0	0	0	0	9
Atopobiaceae	0	2	1	1	0	0	0	1	1	0	0	0	0	0	1	0	0	0	7
uncultured rumen bacterium	0	2	1	1	0	0	1	0	0	1	0	0	0	0	0	0	0	1	7
Enbacteriaceae	2	0	0	0	0	2	0	0	0	0	2	0	0	0	0	0	0	0	6
Staphylococcaceae	1	1	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	6
Clostridiales	0	2	2	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	6
Bifidobacteriaceae	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	3
Family XIII	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	3
Mollicutes RF39	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	3
gut metagenome	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	3
Defluviitaleaceae	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	3
Firmicutes bacterium CAG:822	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	3

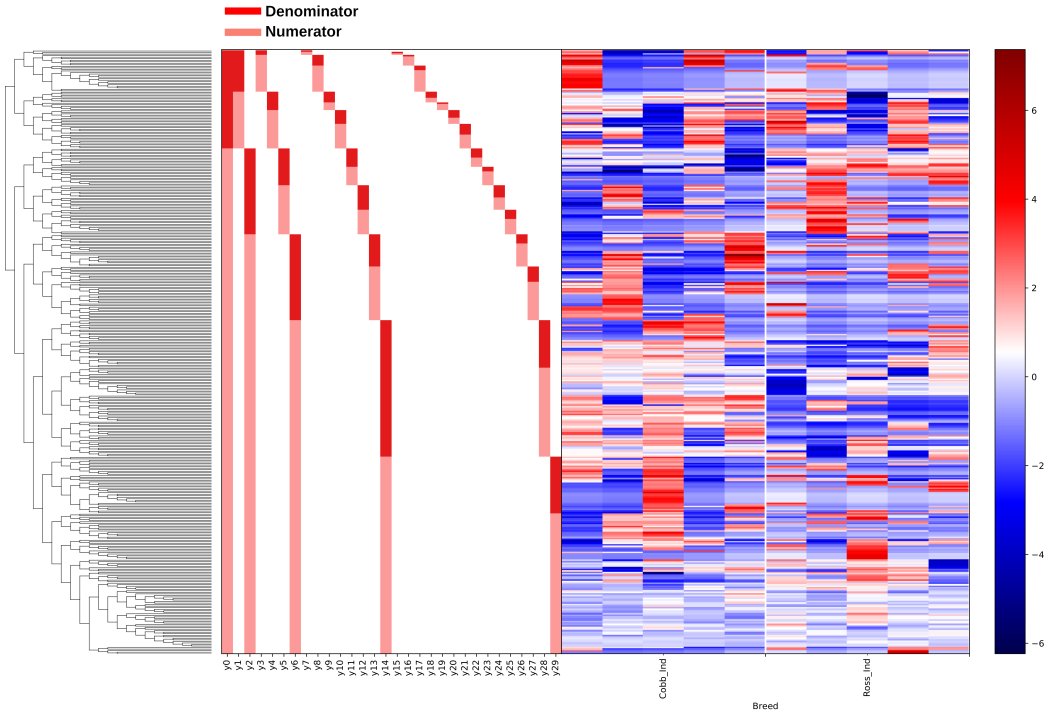
Table A.5: Taxonomy summary at the family level of ASVs in balances that were significantly different between time points in the caecum

Numerator (Num); Denominator (Denom)

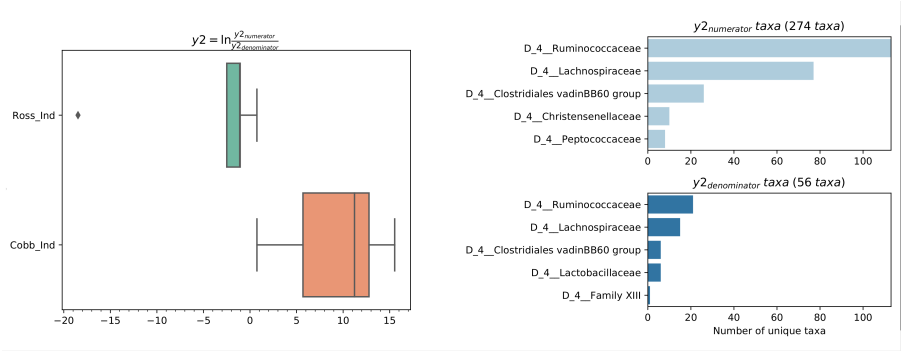
Taxonomy	y2		Total
	Num	Denom	
Ruminococcaceae	83	25	108
Lachnospiraceae	75	20	95
Clostridiales vadinBB60 group	9	0	9
Christensenellaceae	1	5	6
Lactobacillaceae	4	0	4
Burkholderiaceae	4	0	4
Bacillaceae	0	4	4
Bacteroidaceae	3	0	3
Eggerthellaceae	1	2	3
Enterobacteriaceae	2	0	2
Peptostreptococcaceae	1	1	2
Enterococcaceae	1	1	2
Erysipelotrichaceae	1	1	2
uncultured rumen bacterium	1	0	1
Defluviitaleaceae	1	0	1
Staphylococcaceae	1	0	1
Bifidobacteriaceae	1	0	1
Mollicutes RF39	1	0	1
Eubacteriaceae	1	0	1
Clostridiaceae 1	1	0	1
Atopobiaceae	1	0	1
Firmicutes bacterium CAG:822	0	1	1
Coriobacteriaceae	0	1	1
Peptococcaceae	0	1	1

Table A.6: Taxonomy summary at the family level of ASVs in balances that were significantly different between caecal lumen and mucus

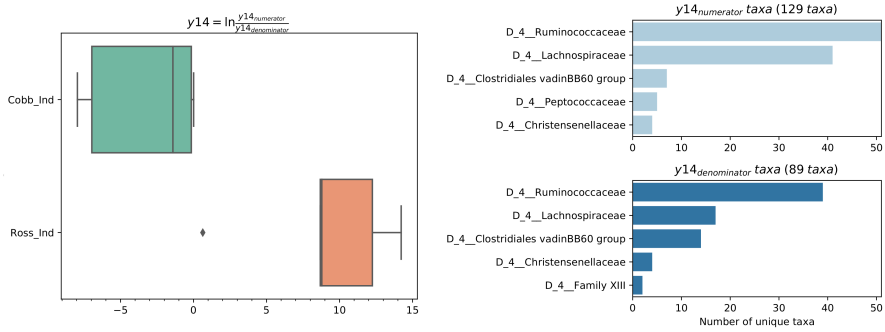
Numerator (Num); Denominator (Denom)



(a) Log abundance of ASVs between Cobb and Ross chickens

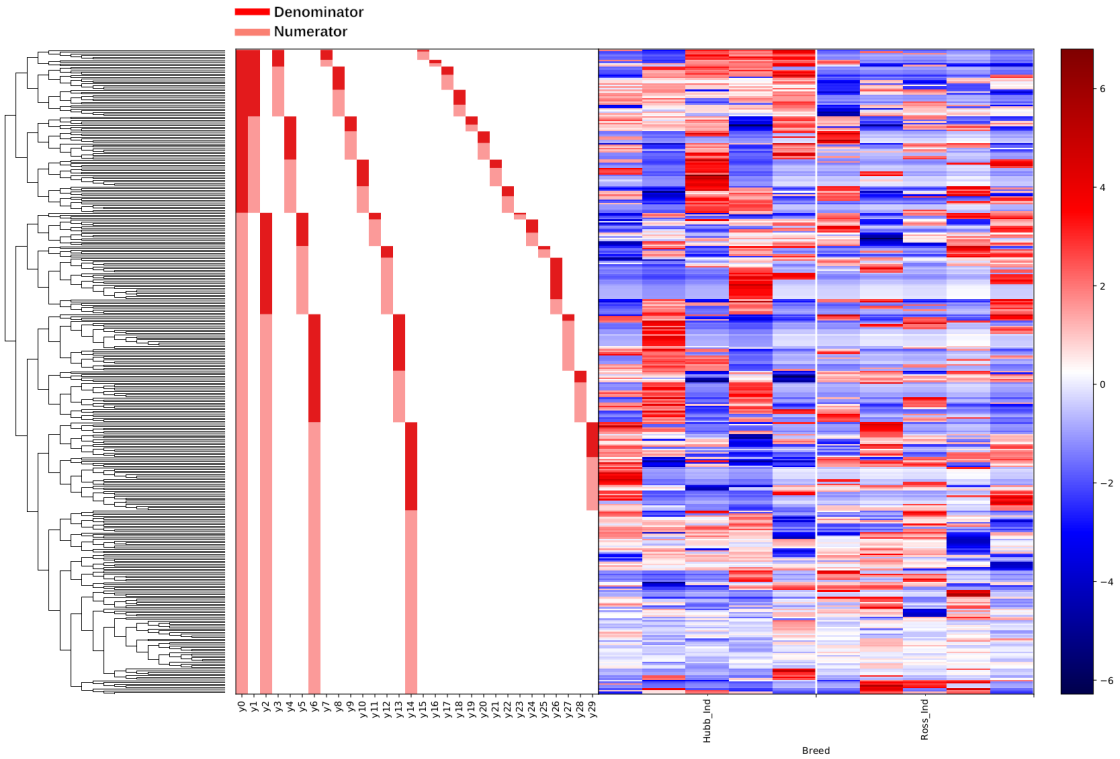


(b) Log ratio and taxonomic composition at the family level of balance y2

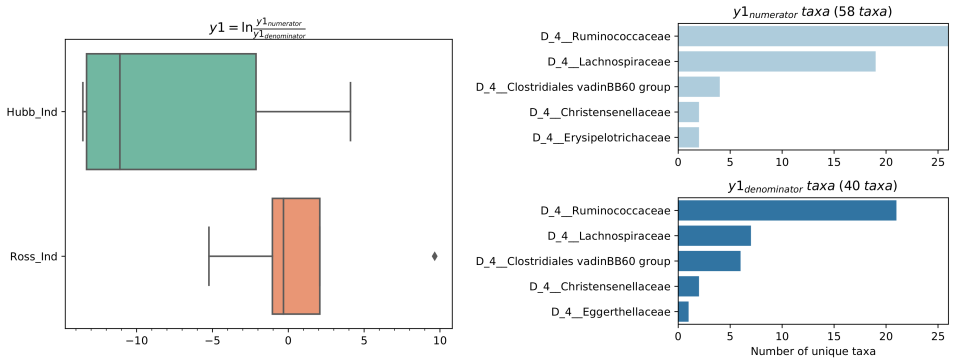


(c) Log ratio and taxonomic composition at the family level of balance y14

Figure A.1: Results of Gneiss analysis comparing the mucus microbiome of Cobb and Ross chickens at 42 d.p.h.

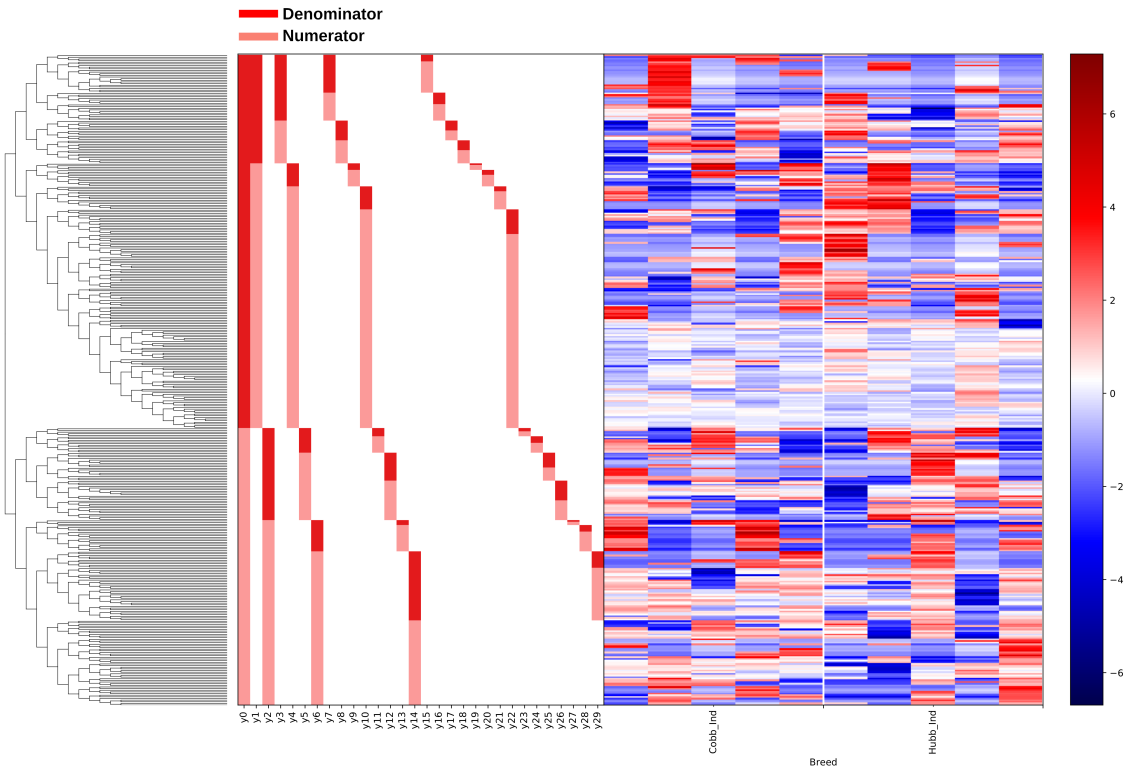


(a) Log abundance of ASVs between Ross and Hubbard chickens

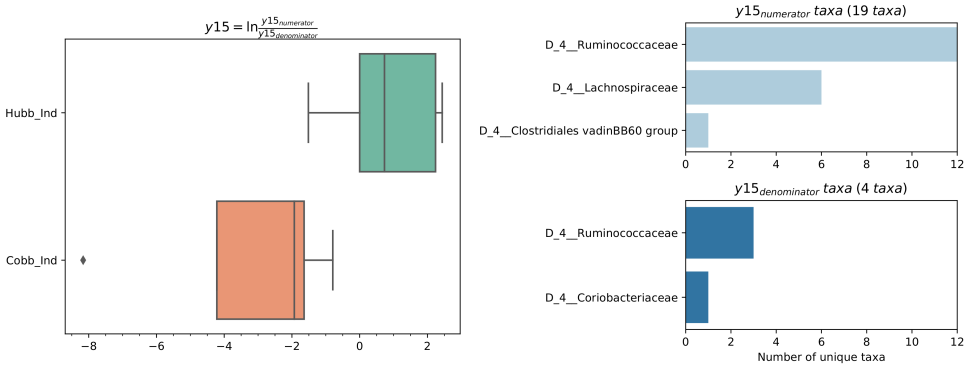


(b) Log ratio and taxonomic composition at the family level of balance y1

Figure A.2: Results of Gneiss analysis comparing the mucus microbiome of Ross and Hubbard chickens at 42 d.p.h.



(a) Log abundance of ASVs between Cobb and Hubbard chickens



(b) Log ratio and taxonomic composition at the family level of balance y15

Figure A.3: Results of Gneiss analysis comparing the mucus microbiome of Cobb and Hubbard chickens at 42 d.p.h.

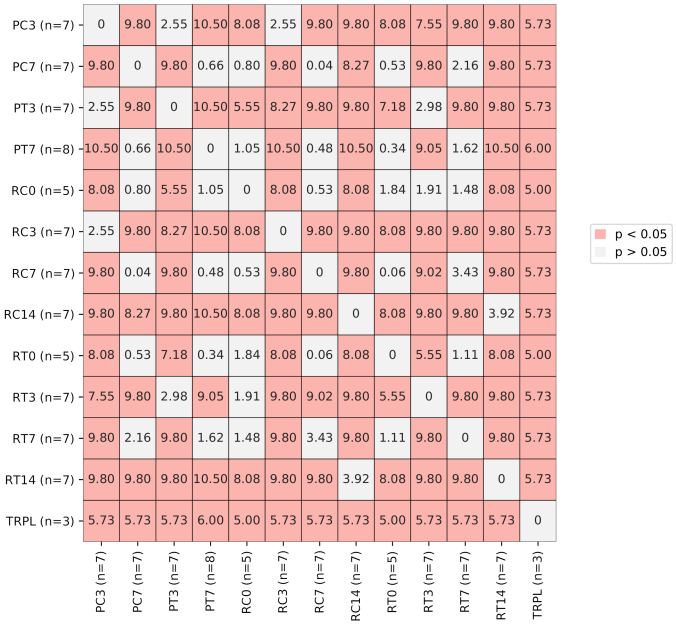
Appendix B

The *In Ovo* Microbiome and Altering the Early Microbiome Using Topical Egg Treatments

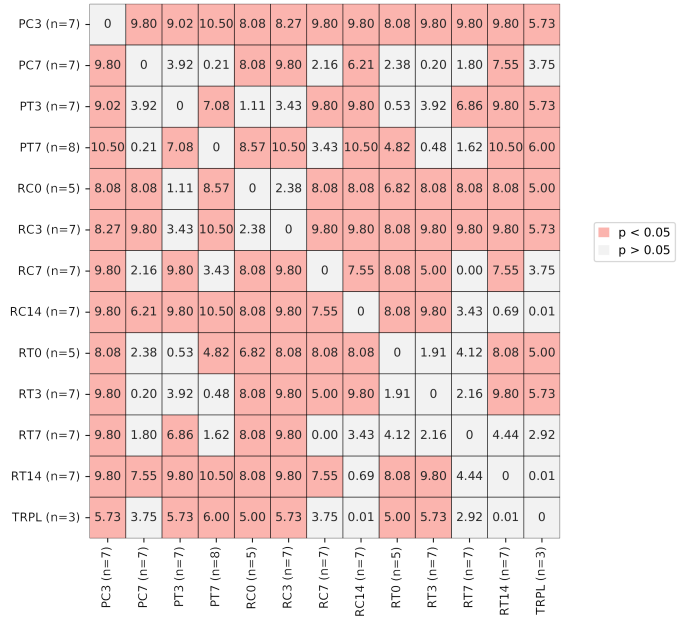
Taxonomy	y0		y2		y5		y6		y14		y27		Total
	Num	Denom	Num	Denom	Num	Denom	Num	Denom	Num	Denom	Num	Denom	
Ruminococcaceae	93	11	83	10	7	3	83	0	83	0	35	48	456
Lachnospiraceae	91	28	65	26	18	8	65	0	65	0	52	13	431
Clostridiaceae 1	19	2	17	2	2	0	11	6	9	2	8	1	79
Erysipelotrichaceae	14	2	5	9	9	0	5	0	5	0	2	3	54
Enterobacteriaceae	10	0	7	3	3	0	7	0	7	0	3	4	44
Peptostreptococcaceae	9	0	4	5	5	0	4	0	4	0	4	0	35
Bacillaceae	6	1	6	0	0	0	6	0	6	0	4	2	31
Clostridiales vadinBB60 group	6	0	6	0	0	0	6	0	6	0	2	4	30
Paenibacillaceae	4	0	3	1	1	0	3	0	3	0	3	0	18
Enterococcaceae	3	0	1	2	0	2	1	0	1	0	1	0	11
uncultured rumen bacterium	2	0	2	0	0	0	2	0	2	0	2	0	10
Lactobacillaceae	2	0	2	0	0	0	2	0	2	0	2	0	10
Microbacteriaceae	1	0	1	0	0	0	1	0	1	0	1	0	5
Christensenellaceae	1	0	1	0	0	0	1	0	1	0	0	1	5
Bacillales	1	0	1	0	0	0	1	0	1	0	1	0	5

Table B.1: Taxonomy summary at the family level of ASVs in balances that were significantly different between caecal samples from treated and control chicks in the pilot experiment

Numerator (Num); Denominator (Denom)



(a) FPD Index



(b) SD Index

Figure B.1: Categorical heatmaps comparing alpha diversity between sample groups

Alpha diversity between groups was compared using a pairwise Kruskal-Wallis test. Significant results between groups are indicated in red. Each box is annotated with the pairwise Kruskal-Wallis test statistic. Significant differences in alpha diversity were noted between treated and control samples at 3 d.p.h (both experiments) and 14 d.p.h (Repeat Experiment). Alpha diversity was significantly higher between control chicks from the pilot experiment and treated chicks from the repeat experiment at 7 d.p.h.

Taxonomy	y0		y4		y10		Total
	Num	Denom	Num	Denom	Num	Denom	
Lachnospiraceae	90	103	101	2	18	83	397
Ruminococcaceae	79	76	76	0	17	59	307
Clostridiaceae 1	1	38	31	5	0	31	106
Erysipelotrichaceae	2	10	10	0	0	10	32
Clostridiales vadinBB60 group	7	8	8	0	1	7	31
Peptostreptococcaceae	3	8	8	0	0	8	27
Enterobacteriaceae	0	8	6	2	2	4	22
Enterococcaceae	0	7	4	3	0	4	18
Bacillaceae	4	3	3	0	0	3	13
Lactobacillaceae	2	3	3	0	1	2	11
Christensenellaceae	1	2	2	0	0	2	7
Microbacteriaceae	0	2	2	0	0	2	6
Staphylococcaceae	0	2	2	0	0	2	6
Paenibacillaceae	0	3	0	0	0	0	3
Alicyclobacillaceae	0	1	1	0	0	1	3
Propionibacteriaceae	0	1	1	0	0	1	3
Burkholderiaceae	0	1	1	0	0	1	3
Thermaceae	0	1	1	0	0	1	3
Hydrogenophilaceae	0	1	1	0	0	1	3
Leuconostocaceae	0	1	1	0	0	1	3
Sanguibacteraceae	0	1	1	0	0	1	3
Moraxellaceae	0	1	1	0	0	1	3
Nocardiaceae	0	1	1	0	0	1	3
Peptococcaceae	0	1	1	0	0	1	3
uncultured rumen bacterium	2	0	0	0	0	0	2
Streptococcaceae	1	0	0	0	0	0	1

Table B.2: Taxonomy summary at the family level of ASVs in balances that were significantly different between caecal samples from treated and control chicks in the repeat experiment

Numerator (Num); Denominator (Denom)

Taxonomy	y0		y2		y9		y11		y13		Total
	Num	Denom	Num	Denom	Num	Denom	Num	Denom	Num	Denom	
Lachnospiraceae	58	0	13	45	42	3	11	2	42	0	216
Ruminococcaceae	31	0	4	27	27	0	4	0	25	2	120
Clostridiaceae 1	25	3	3	22	19	1	1	2	18	1	95
Enterobacteriaceae	14	0	3	11	11	0	3	0	11	0	53
Microbacteriaceae	10	0	0	10	10	0	0	0	10	0	40
Peptostreptococcaceae	11	0	2	9	8	1	0	0	8	0	39
Bacillaceae	9	0	0	9	8	1	0	0	8	0	35
Lactobacillaceae	8	0	0	8	8	0	0	0	8	0	32
Erysipelotrichaceae	7	0	0	7	5	2	0	0	5	0	26
Enterococcaceae	3	2	0	3	3	0	0	0	2	1	14
Nocardiaceae	3	0	0	3	3	0	0	0	3	0	12
Staphylococcaceae	3	0	0	3	3	0	0	0	3	0	12
Pseudomonadaceae	3	0	0	3	3	0	0	0	3	0	12
Moraxellaceae	2	0	0	2	2	0	0	0	2	0	8
Corynebacteriaceae	2	0	0	2	2	0	0	0	2	0	8
Sanguibacteraceae	2	0	0	2	2	0	0	0	2	0	8
Paenibacillaceae	2	0	0	2	2	0	0	0	2	0	8
Propionibacteriaceae	1	0	0	1	1	0	0	0	1	0	4
Christensenellaceae	1	0	0	1	1	0	0	0	1	0	4
Micrococcaceae	1	0	0	1	1	0	0	0	1	0	4
Rhizobiaceae	1	0	0	1	1	0	0	0	1	0	4
Alicyclobacillaceae	1	0	0	1	1	0	0	0	1	0	4
Burkholderiaceae	1	0	0	1	1	0	0	0	1	0	4
Pirellulaceae	1	0	0	1	1	0	0	0	1	0	4
Thermaceae	1	0	0	1	1	0	0	0	1	0	4

Table B.3: Taxonomy summary at the family level of ASVs in balances that were significantly different between ileal samples from treated and control chicks in the pilot experiment

Numerator (Num); Denominator (Denom)

Taxonomy	y0		y2		y5		y11		y21		y22		y26		y28		Total
	Num	Denom	Num	Denom	Num	Denom	Num	Denom	Num	Denom	Num	Denom	Num	Denom	Num	Denom	
Lachnospiraceae	102	0	102	0	0	0	0	0	26	0	56	20	26	0	45	11	388
Ruminococcaceae	43	0	43	0	0	0	0	0	15	0	28	0	15	0	23	5	172
Clostridiaceae 1	40	2	35	5	5	0	0	0	33	0	0	0	30	3	0	0	153
Peptostreptococcaceae	15	6	15	0	0	0	0	0	9	0	6	0	9	0	4	2	66
Enterococcaceae	11	0	9	2	0	2	0	2	8	0	1	0	8	0	1	0	44
Erysipelotrichaceae	10	1	10	0	0	0	0	0	6	0	4	0	6	0	4	0	41
Bacillaceae	10	0	10	0	0	0	0	0	10	0	0	0	10	0	0	0	40
Enterobacteriaceae	9	0	5	4	2	2	2	0	4	0	1	0	4	0	1	0	34
Microbacteriaceae	5	0	5	0	0	0	0	0	2	0	3	0	2	0	3	0	20
Paenibacillaceae	5	0	5	0	0	0	0	0	5	0	0	0	3	2	0	0	20
Lactobacillaceae	4	2	4	0	0	0	0	0	3	0	1	0	3	0	1	0	18
Staphylococcaceae	4	0	4	0	0	0	0	0	4	0	0	0	4	0	0	0	16
Pasteurellaceae	3	0	3	0	0	0	0	0	3	0	0	0	3	0	0	0	12
uncultured rumen bacterium	2	0	2	0	0	0	0	0	1	0	1	0	1	0	0	1	8
Rhizobiaceae	2	0	2	0	0	0	0	0	1	0	1	0	1	0	1	0	8
Pseudomonadaceae	2	0	2	0	0	0	0	0	2	0	0	0	2	0	0	0	8
Sanguibacteraceae	2	0	2	0	0	0	0	0	1	0	1	0	1	0	1	0	8
Planococcaceae	1	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	4
Prevotellaceae	1	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	4
Listeriaceae	1	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	4
Leuconostocaceae	1	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	4
Clostridiales vadinBB60 group	1	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	4
Nocardiaceae	1	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	4
Christensenellaceae	1	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	4
Streptococcaceae	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	3

Table B.4: Taxonomy summary at the family level of ASVs in balances that were significantly different between ileal samples from treated and control chicks in the repeat experiment

Numerator (Num); Denominator (Denom)

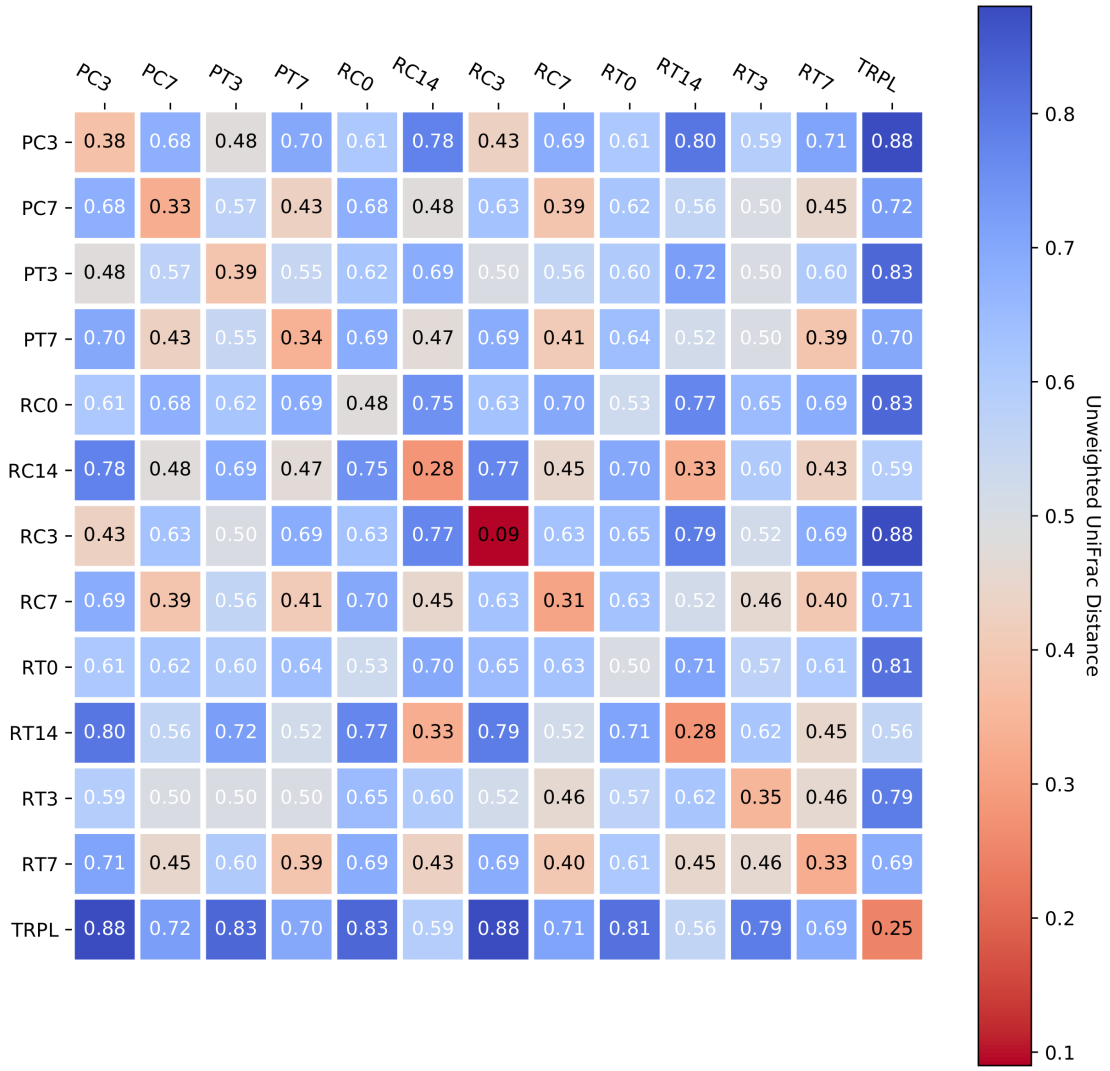
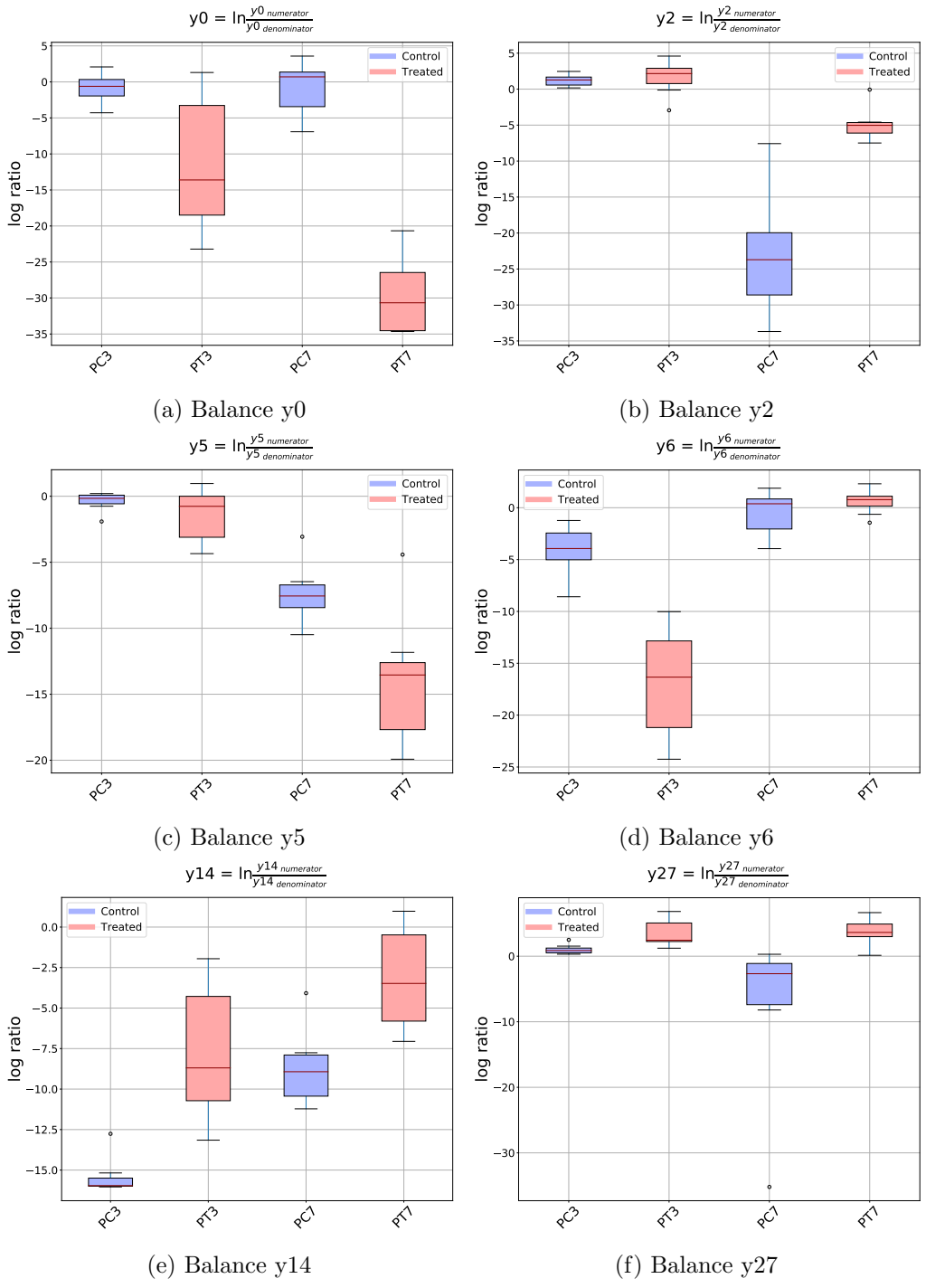


Figure B.2: A heatmap showing the average unweighted UniFrac distance between sample groups

Average unweighted UniFrac distances between sample groups was calculated to evaluate similarities in microbiome composition. A smaller distance between groups indicates increased similarity in microbiome composition. Treated samples were closer to TRPL samples than control samples of a similar age across both experiments with the effect most noticeable at 3 and 7 d.p.h.



(a) Balance y0

(b) Balance y2

(c) Balance y5

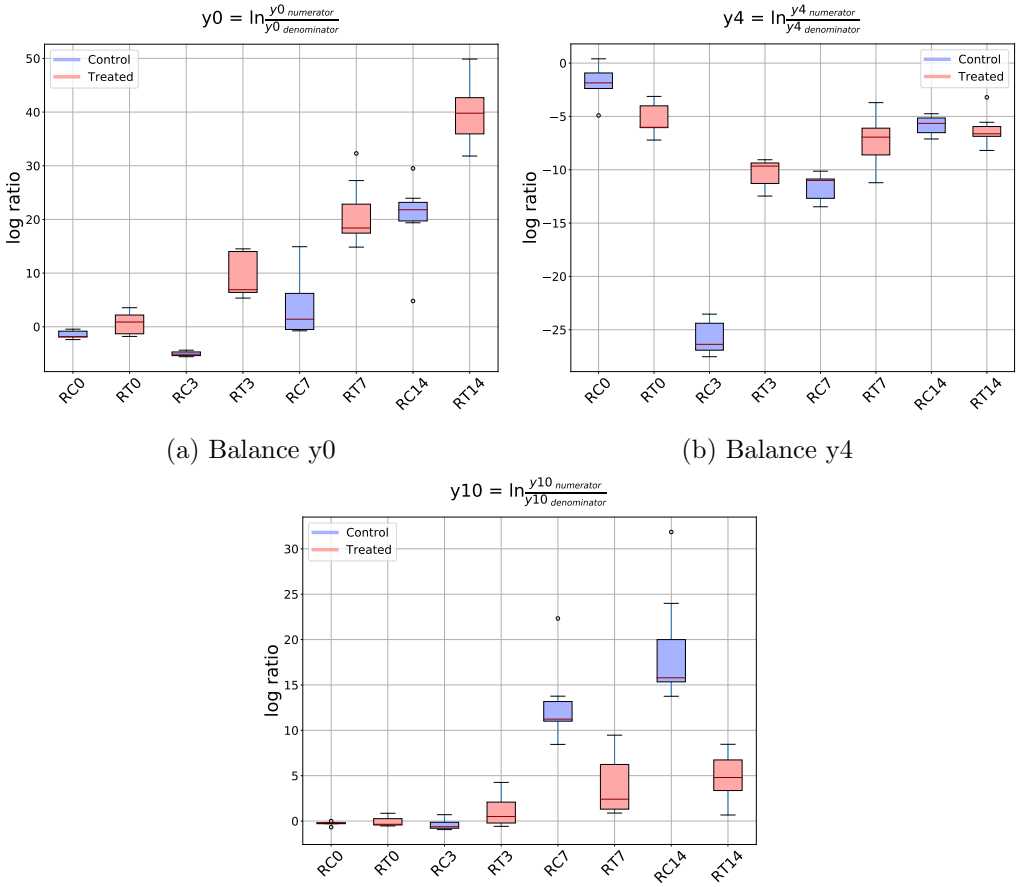
(d) Balance y6

(e) Balance y14

(f) Balance y27

Figure B.3: Log ratios of balances that were significantly different between caecal samples from treated and control chicks in the pilot experiment.

A lower log ratio value suggests a shift in the balance towards denominator taxa either due to decreased abundance of numerator taxa or increased abundance of denominator taxa. In conjunction with the dendrogram heatmap (Figure 3.8) and taxa plot (Figure 3.6), ASVs which are differentially abundant between treated and control chicks were identified and displayed in Table 3.2



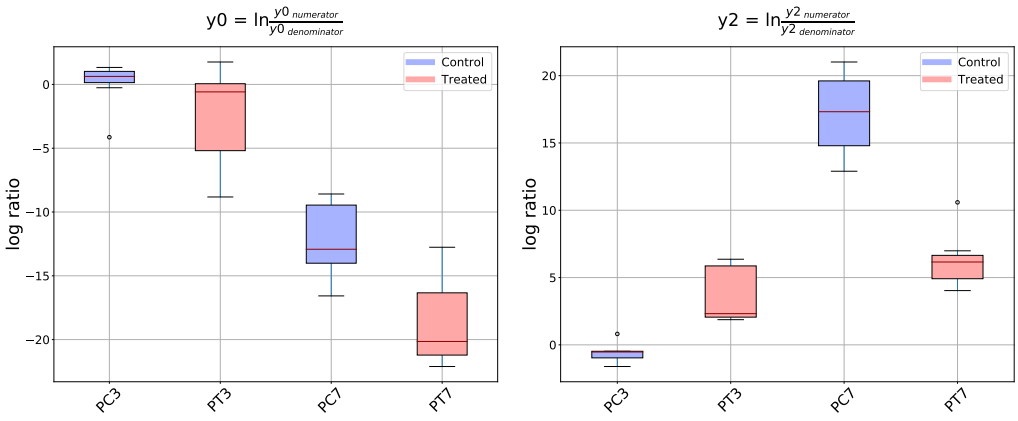
(a) Balance y0

(b) Balance y4

(c) Balance y10

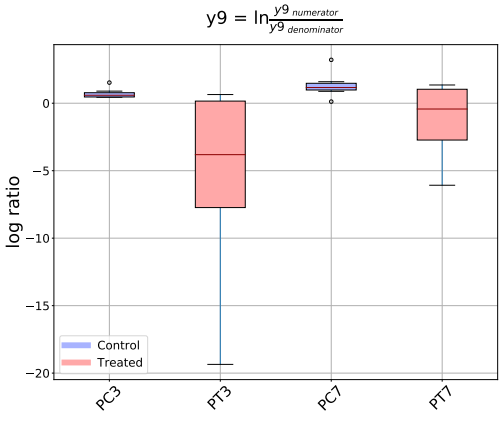
Figure B.4: Log ratios of balances that were significantly different between caecal samples from treated and control chicks in the repeat experiment.

A lower log ratio value suggests a shift in the balance towards denominator taxa either due to decreased abundance of numerator taxa or increased abundance of denominator taxa. In conjunction with the dendrogram heatmap (Figure 3.9) and taxa plot (Figure 3.6), ASVs which are differentially abundant between treated and control chicks were identified and displayed in Table 3.2

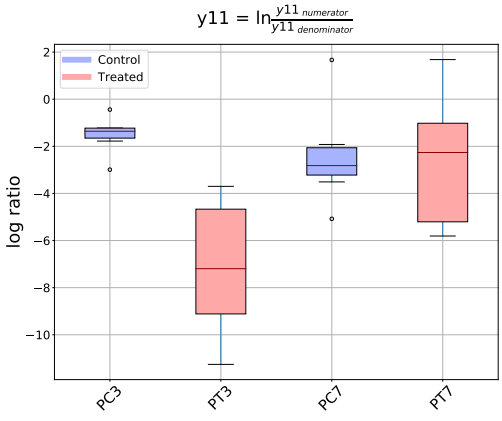


(a) Balance y0

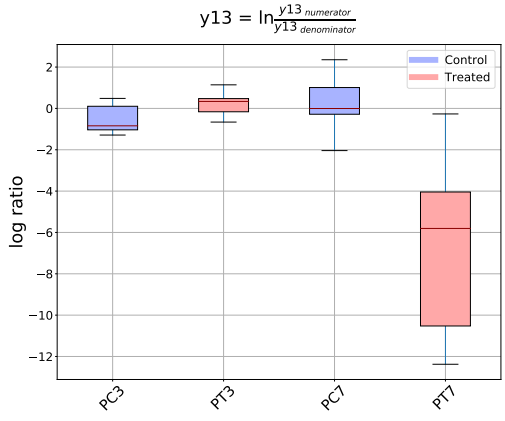
(b) Balance y2



(c) Balance y9



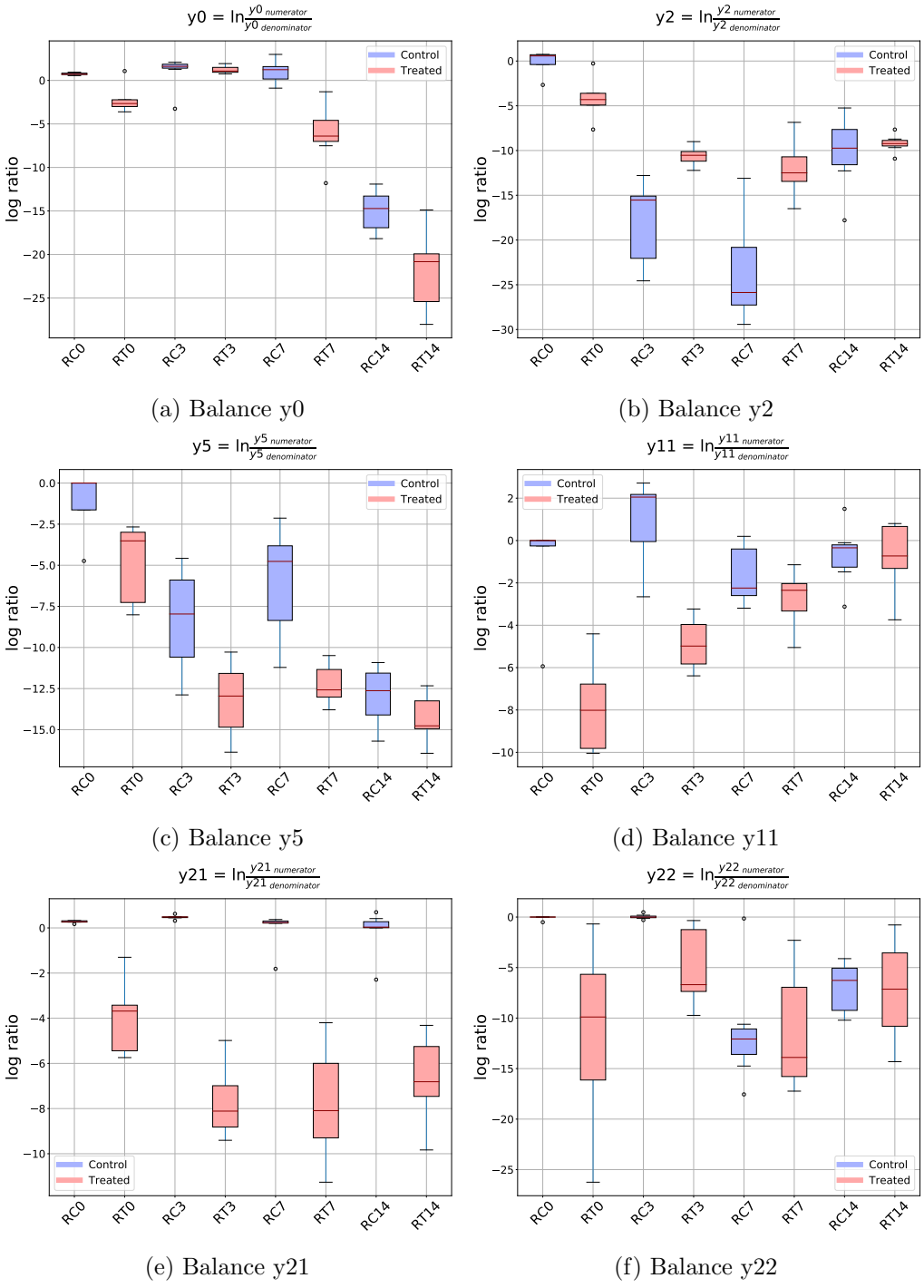
(d) Balance y11



(e) Balance y13

Figure B.5: Log ratios of balances that were significantly different between ileal samples from treated and control chicks in the pilot experiment.

A lower log ratio value suggests a shift in the balance towards denominator taxa either due to decreased abundance of numerator taxa or increased abundance of denominator taxa. In conjunction with the dendrogram heatmap (Figure 3.10) and taxa plot (Figure 3.7), ASVs which are differentially abundant between treated and control chicks were identified and displayed in Table 3.3



(a) Balance y0

(b) Balance y2

(c) Balance y5

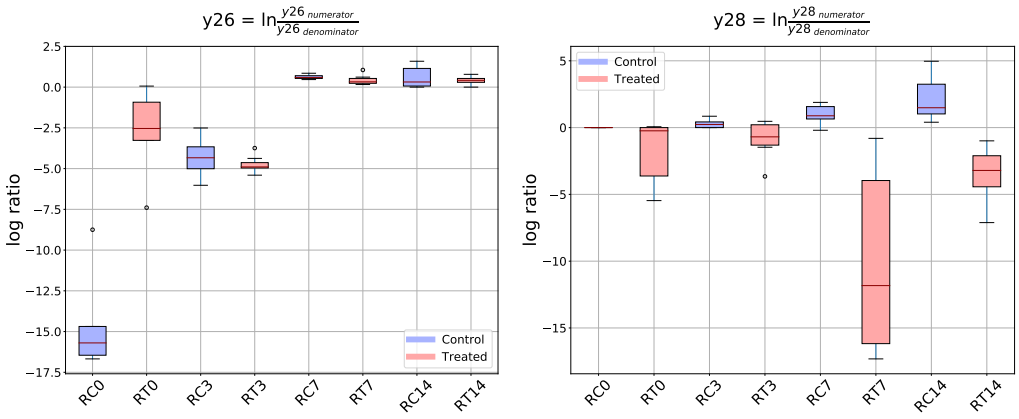
(d) Balance y11

(e) Balance y21

(f) Balance y22

Figure B.6: Log ratios of balances that were significantly different between ileal samples from treated and control chicks in the repeat experiment.

A lower log ratio value suggests a shift in the balance towards denominator taxa either due to decreased abundance of numerator taxa or increased abundance of denominator taxa. In conjunction with the dendrogram heatmap (Figure 3.11) and taxa plot (Figure 3.7), ASVs which were differentially abundant between treated and control chicks were identified and displayed in Table 3.3



(g) Balance y26

(h) Balance y28

Figure B.6: Log ratios of balances that were significantly different between ileal samples from treated and control chicks in the repeat experiment.

Appendix C

Administration of an In-Feed Probiotic To Alter the Caecal Microbiome

DIET FORMULATION AND SPECIFICATION DATA

BASIC DIET INFORMATION:

Code:	996388
Name:	BROILER STARTER (P) BROILER STARTER MEAL (FG)
Date:	23/01/2014

CALCULATED ANALYSIS:

		FRESH	10% H2O
TOTAL	%	100.00	100.00
MOISTURE	%	11.40	10.00
CRUDE OIL	%	4.90	4.98
CRUDE PROTEIN	%	23.24	23.61
CRUDE FIBRE	%	2.72	2.76
ASH	%	5.88	5.97
NFE	%	51.56	52.37
PECTIN	%	1.14	1.16
HEMICELLULOSE	%	5.86	5.95
CELLULOSE	%	2.94	2.99
LIGNIN	%	0.74	0.75
STARCH	%	37.67	38.27
SUGAR	%	3.91	3.97
GROSS ENERGY	MJ/kg	16.55	16.81
DIGESTIBLE ENERGY	MJ/kg	14.73	14.96
METABOLISABLE ENERGY	MJ/kg	13.59	13.80
AF ENERGY	kcal/kg	3432.48	3486.72
C14 1 MYRISTOLEIC	%	0.02	0.02
C16 1 PALMITOLEIC	%	0.13	0.13
C18 1 W9 OLEIC	%	1.19	1.21
C18 2 W6 LINOLEIC	%	2.16	2.19
C18 3 W3 LINOLENIC	%	0.27	0.27
C20 4 W6 ARICHIDONIC	%	0.08	0.08
C22 5 W3 CLUPANODONIC	%	0.00	0.00
C12:0 LAURIC	%	0.02	0.02
C14:0 MYRISTIC	%	0.06	0.06
C16:0 PALMITIC	%	0.52	0.53
C18:0 STEARIC	%	0.13	0.13
ARGININE	%	1.42	1.44
LYSINE	%	1.29	1.31
S LYS	%	0.12	0.12
METHIONINE	%	0.58	0.59
S METH	%	0.18	0.18
CYSTINE	%	0.42	0.43
S CYST	%	0.00	0.00
TRYPTOPHAN	%	0.28	0.28
S TRYPT	%	0.00	0.00

		FRESH	10% H2O
CL	%	0.17	0.17
S CL	%	0.12	0.12
K	%	0.79	0.80
S K	%	0.00	0.00
MG	%	0.15	0.15
S MG	%	0.00	0.00
FE	mg/kg	114.43	116.24
S FE	mg/kg	33.34	33.87
CU	mg/kg	13.08	13.29
S CU	mg/kg	5.00	5.08
MN	mg/kg	106.48	108.16
S MN	mg/kg	79.86	81.12
ZN	mg/kg	82.85	84.16
S ZN	mg/kg	59.98	60.93
CO	µg/kg	344.12	349.56
S CO	µg/kg	287.50	292.04
I	µg/kg	1081.79	1098.88
S I	µg/kg	1016.00	1032.05
SE	µg/kg	239.08	242.86
S SE	µg/kg	150.00	152.37
F	mg/kg	9.17	9.31
VIT A	iu/kg	12605.00	12804.18
S VIT A	iu/kg	10000.00	10158.01
VIT D3	iu/kg	3000.00	3047.40
S VIT D3	iu/kg	3000.00	3047.40
VIT E	iu/kg	23.42	23.79
S VIT E	iu/kg	8.00	8.13
VIT B1 THI	mg/kg	4.82	4.90
S VIT B1	mg/kg	0.81	0.82
VIT B2 RIB	mg/kg	6.18	6.28
S VIT B2	mg/kg	5.04	5.12
VIT B6 PYR	mg/kg	4.08	4.14
S VIT B6	mg/kg	0.82	0.83
VIT B12 CY	µg/kg	8.46	8.59
S VIT B12	µg/kg	8.00	8.13
VIT C ASCO	mg/kg	1.15	1.17
S VIT C	mg/kg	0.00	0.00

HISTIDINE	%	0.60	0.61
THREONINE	%	0.92	0.93
S THREO	%	0.00	0.00
ISOLEUCINE	%	1.05	1.07
LEUCINE	%	1.94	1.97
PHENYLALAN	%	1.25	1.27
VALINE	%	1.15	1.17
TYROSINE	%	0.85	0.86
TAURINE	%	0.00	0.00
GLYCINE	%	0.97	0.99
ASPARTIC A	%	2.03	2.06
GLUTAMIC A	%	4.71	4.78
PROLINE	%	1.54	1.56
SERINE	%	1.19	1.21
HYD PROLIN	%	0.01	0.01
HYD LYSINE	%	0.00	0.00
ALANINE	%	0.91	0.92
CA	%	0.94	0.95
S CA	%	0.83	0.84
TOTAL P	%	0.71	0.72
S PHOS	%	0.35	0.36
PHYTATE P	%	0.21	0.21
AVAIL P	%	0.50	0.51
NA	%	0.17	0.17
S NA	%	0.08	0.08

VIT K MENE	mg/kg	1.31	1.33
S VIT K	mg/kg	1.03	1.05
FOLIC ACID	mg/kg	1.53	1.55
S FOLIC	mg/kg	0.95	0.97
NICOTINIC	mg/kg	53.10	53.94
S NICOTIN	mg/kg	19.70	20.01
PANTOTHENI	mg/kg	20.74	21.07
S PANTOTH	mg/kg	9.96	10.12
CHOLINE	mg/kg	967.09	982.37
S CHOLINE	mg/kg	76.29	77.50
INOSITOL	mg/kg	2138.50	2172.29
S INOSITOL	mg/kg	0.00	0.00
BIOTIN	µg/kg	231.22	234.87
S BIOTIN	µg/kg	50.00	50.79

SUPP = Supplemented nutrients from manufactured and mined sources. The TOTAL nutrient level including theoretical natural contribution for the diet pre-processing is found immediately above the SUPP nutrient.

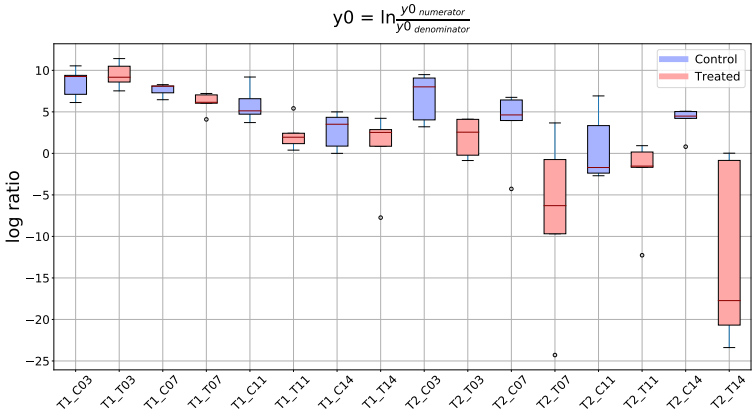
INGREDIENTS:

NAME
WHEAT
MAIZE
DEHULLED EXTRACTED TOASTED SOYA
WHEAT GLUTEN MEAL
SOYA OIL
POTATO PROTEIN
WHEATFEED
FULL FAT TOASTED SOYA
MAIZE GLUTEN MEAL
DICALCIUM PHOSPHATE
VITAMIN & MINERAL MIX
CALCIUM CARBONATE
SODIUM CHLORIDE
SODIUM BICARBONATE
DL-METHIONINE
L-LYSINE

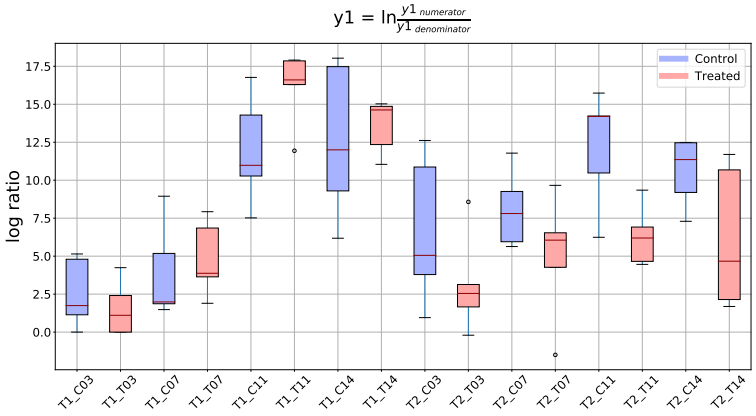
Taxonomy	y0		y1		y6		y12		Total
	Num	Denom	Num	Denom	Num	Denom	Num	Denom	
Lachnospiraceae	25	15	4	11	6	11	2	2	76
Ruminococcaceae	17	16	4	12	4	9	3	1	66
Enterobacteriaceae	10	0	0	0	7	1	2	0	20
Clostridiaceae 1	9	0	0	0	9	0	0	0	18
Enterococcaceae	3	0	0	0	3	0	0	0	6
Lactobacillaceae	3	0	0	0	2	1	0	0	6
Erysipelotrichaceae	1	2	0	2	0	0	0	1	6
Paenibacillaceae	2	0	0	0	2	0	0	0	4
Clostridiales vadinBB60 group	1	1	1	0	1	0	0	0	4
Peptostreptococcaceae	1	0	0	0	0	0	0	1	2
Streptococcaceae	1	0	0	0	0	1	0	0	2

Table C.1: Taxonomy summary at the family level of ASVs in balances that were significantly different between caecal samples from treated and control chicks in Trials One and Two

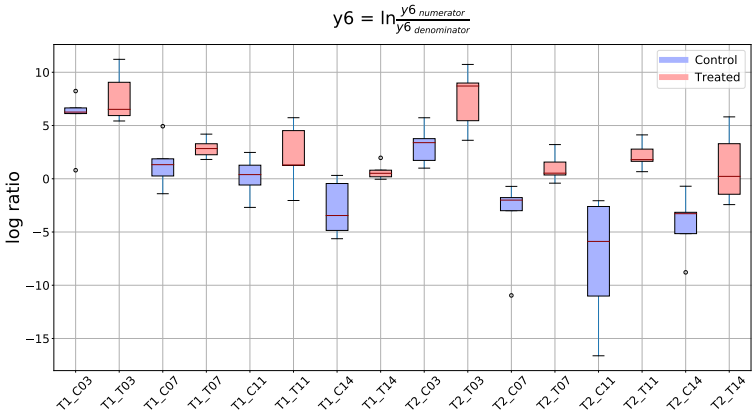
Numerator (Num); Denominator (Denom)



(a) Balance y0



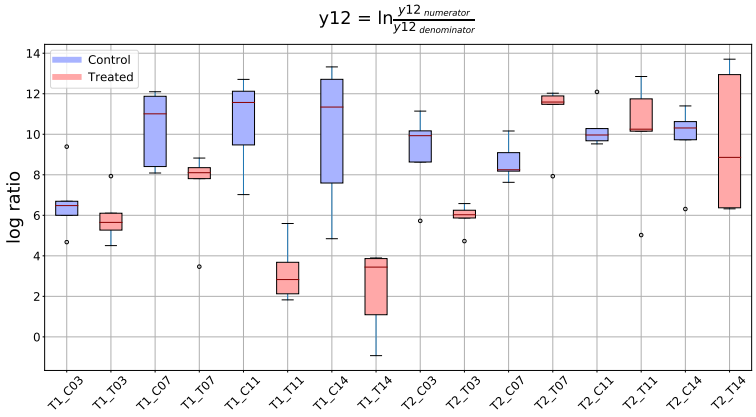
(b) Balance y1



(c) Balance y6

Figure C.1: Log ratios of balances that were significantly different between caecal samples from treated and control chicks in Trials One and Two

A lower log ratio value suggests a shift in the balance towards denominator taxa either due to decreased abundance of numerator taxa or increased abundance of denominator taxa. In conjunction with the dendrogram heatmap (Figure 4.5) ASVs which were differentially abundant between treated and control chicks were identified and displayed in Table 4.2



(d) Balance y12

Figure C.1: Log ratios of balances that were significantly different between caecal samples from treated and control chicks in Trials One and Two